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DISSERTAÇÃO DE MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Biodegradation of fluorinated compounds widely used in agro-industrial applications

Diogo Alves da Mota Alexandrino

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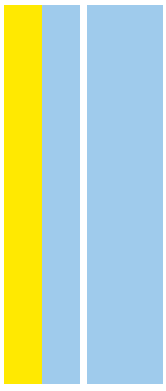
2016



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BIODEGRADATION OF FLUORINATED COMPOUNDS WIDELY USED IN AGRO-INDUSTRIAL CONTEXTS

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Maria F. Carvalho, without whom the work integrated in this thesis would have not been possible. I genuinely thank her incredible dedication and trust, certain that part of my future goals have been established as a result of her mentoring, which became both an incredible honour and a fundamental phase in my personal and professional development.

I would also like to thank my co-supervisor, Dr. Ana Paula Mucha, to whom I thank for the opportunity of integrating her laboratory, where I was always given all the conditions to develop my work to the fullest of its potential.

Secondly, I would like to acknowledge CIIMAR - Interdisciplinary Centre of Marine and Environmental Research and Departamento de Química e Bioquímica of Faculty of Sciences of University of Porto, for the use of all the equipment, installations and facilities.

To my lab mates at Ecobiotec (CIIMAR-UP), I recognise their friendship, as well as all the input in my work and precious help and support, with a special emphasis to Patricia Duarte, Filipa Santos, Joana Fernandes and Inês Ribeiro.

I am also grateful for the involvement of Dr. Marisa Almeida, Dr. Filipe Pereira and Prof. Rui Oliveira in my work: to Dr. Marisa Almeida, I thank her support in all HPLC analysis, as well as the input in the scientific revision in one of the submitted scientific manuscripts that are part of this thesis; to Dr. Filipe Pereira for his incredible support and input regarding the molecular biology tools used in this work; to Prof. Rui Oliveira for his mentorship since my Bachelor Degree and for aiding on the revision of part of this thesis.

Finally, I would like to give my deepest regards to my family and close friends, acknowledging their friendship, support and company during this stage of my life. Especially to my parents, I thank them for always enabling and supporting me, shaping the person I am today.

The research contemplated in this thesis was supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF),

in the framework of the programme PT2020, by the structured Program of R&D&I INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources, reference NORTE-01-0145-FEDER-000035, namely within the Research Line ECOSERVICES (Assessing the environmental quality, vulnerability and risks for the sustainable management of the NW coast natural resources and ecosystem services in a changing world) within the R&D Institution CIIMAR (Interdisciplinary Centre of Marine and Environmental Research), supported by the Northern Regional Operational Programme (NORTE2020), through the European Regional Development Fund (ERDF), and by Investigador FCT program supported by FCT, FSE and Programa Operacional Potencial Humano, within the scope of the project IF/00791/2013/CP1197/CT0002.

ABSTRACT

Fluoroorganic compounds are a class of chemicals that are thriving in virtually all economic sectors, essentially due to the unique properties of the fluorine atom. The pharmaceutical and agrochemical industries are two important sectors where these compounds are used, with a wide range of commercial drugs and pesticides belonging to this class of compounds. The aim of this thesis was to investigate the biodegradation of fluoroorganics with distinct chemical structures (aliphatic and aromatic) and applications.

In the first experimental work, the biodegradation of a group of structurally related aliphatic carboxylic fluoroorganics – mono- (MFA), di- (DFA) and trifluoroacetate (TFA) - was investigated, using a variety of environmental samples as a microbial source. Biodegradation experiments were carried out under different modes of substrate supplementation, which included (i) fluoroacetates (FAs) fed as sole carbon source; (ii) FAs (only for DFA and TFA) fed in co-metabolism with sodium acetate and (iii) mixtures of MFA with DFA or TFA. Biodegradation of the target compounds was assessed through fluoride ion release. The results obtained revealed that from the three FAs fed, only MFA was completely defluorinated, while DFA and TFA were recalcitrant in all tested conditions. When present in mixture, DFA was shown to inhibit biodegradation of MFA, whereas TFA had no effect. A total of 15 bacterial isolates were found to degrade as single strains 20 mg L^{-1} of MFA as sole carbon source. 16S rRNA gene sequencing analysis indicated that from these degrading bacteria, only *Delftia acidovorans* had been previously reported to degrade MFA. This work shows that biodegradation of the three tested FAs is very distinct, despite these compounds being structurally related, and draws the attention to the unknown impacts that the accumulation of DFA and TFA may have in the environment as a result of their high recalcitrance.

In the second experiment, biodegradation of a veterinary antibiotic, enrofloxacin (ENR), was investigated both individually and in mixture with a non-fluorinated antibiotic, ceftiofur (CEF). Biodegradation was investigated for a concentration range between 1-3 mg L^{-1} and using acetate as a co-substrate. Microbial inocula were obtained from rhizosphere sediments of plants derived from experimental constructed wetlands designed for the treatment of livestock wastewaters

contaminated with trace amounts of these antibiotics. Complete removal of CEF from the inoculated culture medium was always observed, independently of its concentration or the concomitant presence of ENR. Biodegradation of ENR decreased with the increase in its concentration in the culture medium, with defluorination percentages decreasing from ca. 80 to 4 % in the cultures fed with 1 and 3mg L^{-1}, respectively. Ciprofloxacin and norfloxacin were detected as biodegradation intermediates of ENR degradation in the inoculated culture medium supplemented with this antibiotic, indicating that defluorination of at least part of ENR in these cultures is not an immediate catabolic step. Abiotic mechanisms showed to have a high influence in the removal of CEF, affecting less ENR degradation. The enrichment process with the target antibiotics led to significant shifts in the structure and diversity of the microbial communities, predominantly selecting microorganisms belonging to the phyla *Proteobacteria* (e.g. genera *Achromobacter, Variovorax* and *Stenotrophomonas*) and *Bacteroidetes* (e.g. genera *Dysgonomonas*, *Flavobacterium* and *Chryseobacterium*). The results presented in this study indicate that biodegradation can be an important mechanism for the environmental removal of the tested compounds. In overall, the two developed works indicate that fluorinated compounds are a challenge for microbial degradation yet, due to the high metabolic versatility of microorganisms, biodegradation is still a possible mechanism for their environmental remediation. The results obtained in the present thesis also indicate that the degree of fluorination and compound concentration have a crucial role in the recalcitrance of fluorinated compounds.

RESUMO

Os compostos organofluorados constituem uma classe de compostos químicos cuja utilização se encontra em expansão em praticamente todos os setores económicos, essencialmente devido às propriedades únicas do átomo de flúor. Os setores agroquímico e farmacêutico constituem dois segmentos industriais onde esta classe de compostos tem especial relevância, dado o elevado número de produtos farmacêuticos e pesticidas fluorados atualmente comercializados. O objetivo desta dissertação foi investigar a biodegradação de compostos orgânicos fluorados com distintas estruturas químicas (alifáticos e aromáticos) e aplicações práticas.

No primeiro trabalho experimental investigou-se a biodegradação de três compostos fluorados alifáticos estruturalmente semelhantes – mono- (MFA), di- (DFA) e trifluoroacetato (TFA) – utilizando como inóculos, microrganismos provenientes de diferentes amostras ambientais. Nas experiências de biodegradação realizadas, os fluoroacetatos (FAs) foram suplementados de diferentes modos: (i) FAs como fonte única de carbono; (ii) DFA ou TFA em cometabolismo com acetato e (iii) misturas de MFA com DFA ou TFA. A libertação do ião fluoreto foi utilizada como indicador da biodegradação dos FAs. Os resultados obtidos revelaram que dos três FAs alimentados apenas o MFA foi completamente defluorinado, enquanto o DFA e TFA foram recalcitrantes em todas as condições testadas. Quando em mistura, a presença de DFA inibiu a biodegradação de MFA, enquanto o TFA não teve qualquer efeito inibitório. Um total de 15 isolados bacterianos mostraram ser capazes de degradar individualmente 20 mg L^{-1} de MFA como fonte única de carbono. A sequenciação do gene 16S rRNA desses isolados revelou que apenas a espécie *Delftia acidovorans* foi anteriormente reportada como degradadora de MFA. Estes resultados mostram que a biodegradação destes três FAs é bastante distinta, apesar das suas similaridades estruturais, e chamam a atenção para a importância de conhecer os impactos decorrentes da persistência e acumulação de DFA e TFA no ambiente, como resultado da elevada recalcitrância destes compostos.

Na segunda experiência, estudou-se a biodegradação de um antibiótico veterinário, enrofloxacina (ENR), suplementado individualmente e em mistura com um antibiótico não fluorado, ceftiofur (CEF). A biodegradação foi investigada para uma gama de concentrações entre 1-3 mg L^{-1}, utilizando acetato como co-substrato. Utilizou-se como inóculo, rizosedimento de plantas provenientes de uma fito-etar experimental desenhada para o tratamento de efluentes de pecuária contaminados

com concentrações vestigiais dos antibióticos estudados. A completa remoção de CEF foi sempre observada, independentemente da sua concentração nas culturas microbianas ou da concomitante presença de ENR. A biodegradação de ENR diminuiu com o aumento da sua concentração no meio de cultura, com percentagens de defluorinação oscilando entre os 80 e os 4 % nas culturas suplementadas com 1 e 3 mg L^{-1}, respetivamente. Os intermediários metabólicos ciprofloxacina e norfloxacina foram detetados nas culturas suplementadas com ENR, indicando que pelos menos parte da molécula de ENR não é imediatamente sujeita a uma reação de defluorinação. Os mecanismos abióticos mostraram ter uma grande influência na remoção de CEF, não afetando de forma tão acentuada a degradação de ENR. O processo de enriquecimento com os antibióticos estudados levou a alterações significativas ao nível da estrutura e diversidade das comunidades microbianas, selecionando predominantemente microrganismos pertencentes aos filos *Proteobacteria* (p. ex. géneros *Achromobacter, Variovorax* e *Stenotrophomonas*) e *Bacteroidetes* (p. ex. géneros *Dysgonomonas, Flavobacterium* e *Chryseobacterium*). Os resultados deste estudo mostraram que a biodegradação pode ser um importante mecanismo na remoção destes antibióticos do ambiente.

De uma forma geral, ambos os trabalhos realizados mostram que os compostos orgânicos fluorados constituem um desafio para a degradação microbiana, no entanto, atendendo à elevada versatilidade metabólica dos microrganismos, a biodegradação destes compostos revela-se um mecanismo viável para a sua remediação ambiental. Os resultados obtidos indicam também que tanto o grau de fluorinação como a concentração do composto têm um papel fundamental na recalcitrância dos compostos fluorados.

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ABBREVIATIONS AND SYNCRONIMS

Å - Ångstrom

Bp – Base pair

CEF – Ceftiofur

C-F – Carbon-fluorine

CIP – Ciprofloxacin

CP – Cephalosporins

DFA - Difluoroacetate

ENR – Enrofloxacin

FAdH – Fluoroacetate dehalogenase

FAs - Fluoroacetates

FQ – Fluoroquinolones

HCFC - Hydrochlorofluorocarbon

HFC – Hydrofluorocarbon

Kj – Kilojoule

LOD – Analytical Limit of Detection

LOQ – Analytical Limit of Quantification

MFA - Monofluoroacetate

MM – Minimal medium

NOR – Norfloxacin

OD – Optical Density

OTU – Operational Taxonomic Unit

PCA – Plate-Count Agar

PCR – Polymerase Chain Reaction

Pm - Picometer

QIIME – Quantitative Insights into Microbial Ecology

rRNA – Ribosomal RNA

STE – Sodium Chloride-Tris-EDTA

TFA - Trifluoroacetate

TISAB III – Total Ionic Strength Adjusting Buffer

WWTP – Wastewater Treatment Plant

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CHAPTER I

INTRODUCTION

1. Xenobiotics in the environment

The advances in chemical synthesis have led to the introduction of countless new compounds in all segments of contemporary societies and to the generation of novel products and materials. Today, more than ever, the creation, production, marketing and overall use of novel synthetic and semi-synthetic products happens in an unequalled rate.

While industrialization has been the main drive towards the high standards of living that exist nowadays, this phenomenon is also the main responsible for the environmentally-threatened Earth that we live today. As a result of the rapid industrial development that occurred after the first Industrial Revolution, which was accompanied by a hasty urbanization and an increase on world population, a significant anthropogenic pressure in every component of the environment has been occurring.

The production, introduction and spreading of xenobiotics in the environment is a direct cause of the increased anthropogenic footprint in the environment. Being compounds foreign to nature, xenobiotics have an increased potential for ecosystem damage, attending to their capability of disrupting the dynamics of nature. Moreover, the recycling and natural removal of such products from environmental matrices is not always possible due to their foreign nature and constant environmental input.

2. Fluoroorganic compounds

The first reported synthesis of a fluoroorganic compound dates back to mid-18th century, but it was only in the 1930s that these products gained an industrial dimension with the production of chlorofluorocarbons and other industrially relevant fluorinated products (Okazoe, 2009; Kirsch, 2013). With the development of new methodologies enabling a more efficient synthesis of the carbon-fluorine (C-F) bond, the overall manufacturing of synthetic organofluorines skyrocketed.

In addition to industrial applications, the unique properties of fluoroorganics made these compounds also attractive for other types of applications. For example, the discovery of the first fluorinated pharmaceutical by Heidelberger et al. (1957) (5-fluorouracil, an anticancer drug) drew attention to the role that fluoroorganic compounds could have on the design of pharmaceuticals and agrochemicals.

Nowadays, this class of compounds has a widespread use in various applications, ranging from pharmaceuticals, agrochemicals and biocides, industrial reagents, solvents, anti-adherents, plastics, fire retardants, refrigerants, anaesthetics, among others (Key et al., 1997; Kiel and Engesser, 2015). As a result of this growing use in most economical sectors, the environmental presence of organofluorine compounds has witnessed a proportional increase (Fig. 1) (Key et al., 1997; Kiel and Engesser, 2015).

Biological production of fluorinated molecules is very rare in nature. Biogenic halogenation is verified in *ca.* 3700 organic molecules, but only about 20 of these correspond to fluorinated structures (Gribble, 2003; Kiel and Engesser, 2015). All the known naturally-produced organofluorines are monofluorinated, which contrasts with synthetic fluoroorganic compounds which usually have more than one fluorine atom in their molecules (Key et al., 1997). Thus, the overwhelming majority of organofluorinated compounds are foreign to nature, being xenobiotics, by definition. In addition, the scarce occurrence of natural fluorinated structures indicates that fluorinated compounds do not have a central role in biological processes (Kirsch, 2013).

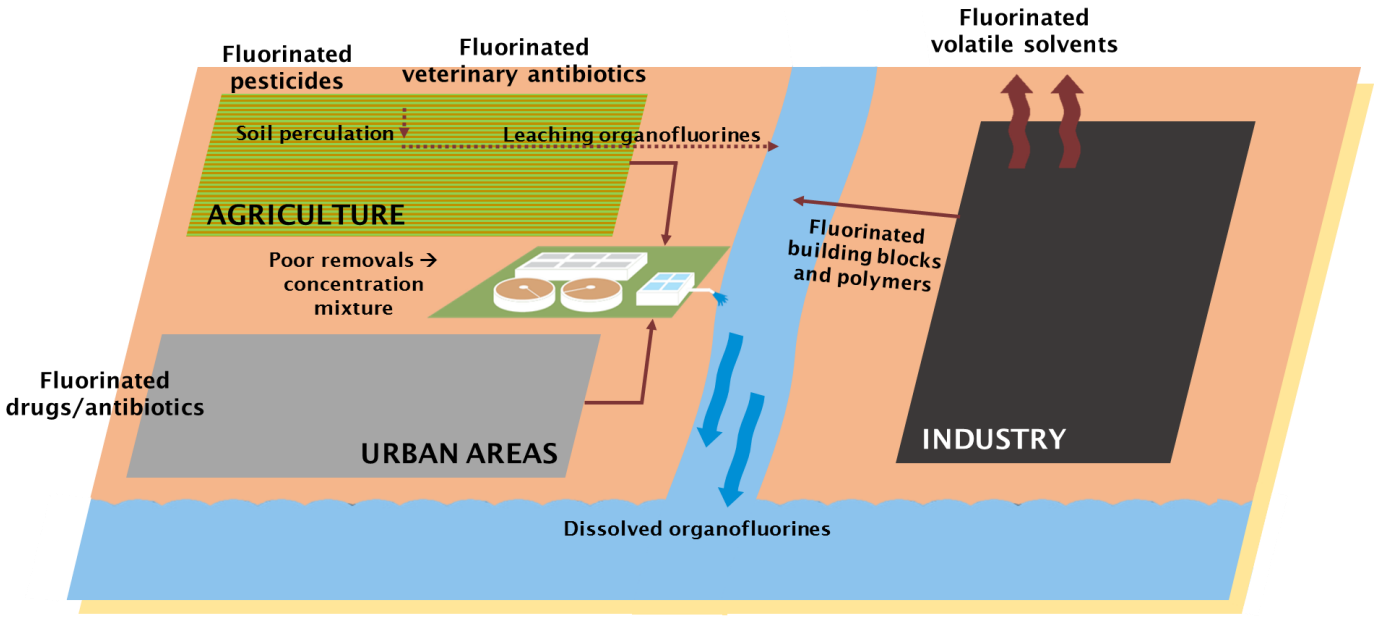


Figure 1. Main anthropogenic sources of organofluorine contaminants and their corresponding environmental dynamics.

2.1. Elemental fluorine

Fluorine is a chemical element belonging to the halogens group. It was first discovered in 1810 by André-Marie Ampére, but the isolation of this element was only achieved in 1886 by Henry Moissan (Chang, 2010). Despite its early discovery, fluorine only became relevant almost a century later (Okazoe, 2009).

Although all halogens are highly reactive, fluorine exhibits an unprecedented reactivity, as it is highly oxidizing and prone to radical formation (Jaccaud et al., 2000; Kirsch, 2013). Also, this element has an extreme electronegativity (and, consequently, a very high ionization energy), which further contributes to its high reactivity (Chang, 2010; Kirsch, 2013).

Due to its peculiar properties and widespread uses, fluorine has been acknowledged has the "*small atom with a big ego*" (Uneyama, 2007). In fact, this element has unique physicochemical properties (Table 1), which justify its current diversified applications. However, these properties also render all fluorinated molecules – either organic or inorganic – a certain outlandishness in terms of structure, reactivity and overall biotic and abiotic behaviour.

The ionic form of fluorine, fluoride, has a very small ionic radius (Table 1), similar to a hydroxyl anion or a hydrogen, meaning that the replacement of a hydrogen atom or a hydroxyl group by fluorine occurs with minimal steric interferences (Jaccaud et al., 2000). Also, fluorine is capable of establishing with carbon one of the strongest chemical bonds known in organic chemistry.

When compared to the other halogens, fluorine is the most abundant, being also one of the most common elements in the planet (Jaccaud et al., 2000). However, this element occurs mainly in inorganic forms, integrating various minerals (fluorspar, fluorite, fluorapatite, cryolith and topaz) (Harnisch and Eisenhauer, 1998). In fact, the natural occurrence of fluorine embedded in organic molecules is a very rare phenomenon.

2.2. The C-F bond

The peculiar properties of organofluorinated compounds can be partially attributed to the special nature of the chemical bond that fluorine establishes with carbon in organic molecules.

This bond is thought to be one of the strongest in organic chemistry, partially due to the high electrostatic attraction between fluorine and carbon and to the excellent orbital compatibility between these two elements (Banks et al., 1994; O'Hagan, 2008; Kirsch, 2013). In addition, due to the extreme electronegativity of the fluorine atom, when fluorine is bonded to carbon it always attracts more strongly the shared electrons, creating a highly polarized chemical bond.

Table 1. Physicochemical properties of the different halogens

Property	**Fluorine**	**Chlorine**	**Bromine**	**Iodine**	**Ref.**
Melting point (ºC)	-223	-102	-7	114	Chang (2010)
Boiling point (ºC)	-187	-35	59	184	Chang (2010)
Atomic radius (*pm*)	72	99	114	140	Chang (2010)
Ionic radius (*pm*)	133	181	196	216	Haynes (2014)
Ionization energy (kJ mol^{-1})	1680	1251	1139	1009	Chang (2010)
Electronegativity (Pauling Scale)	4.0	3.0	2.8	2.7	Chang (2010)
Bond strength when bounded to carbon (kJ mol^{-1})	485	339	285	213	Banks et al. (1994)

Besides, the C-F bond also has a small length (1.35 Å), being only compared to carbon-hydrogen (1.09 Å) and carbon-oxygen bonds (1.43 Å). In fact, the C-F bond possibly represents the smallest chemical bond between carbon and a heteroatom in organic molecules.

As a result of the properties of the C-F bond, fluorinated molecules are less likely to interact with neighbouring molecules (Murray-Rust et al., 1983), with this property closely influencing pharmacokinetics and environmental dynamics of these compounds.

2.3. Biological significance of fluoroorganics

Fluorine-substituted molecules have a high potential as biologically active compounds in areas ranging from medicinal chemistry to agriculture (Ojima, 2013). The applications of organofluorine compounds as therapeutics, diagnostic agents, pesticides, among others, are rapidly expanding, much due to the special properties of these compounds. One clear example of this is demonstrated by the large number of fluorinated pharmaceuticals currently approved for human and veterinary use (Bégué and Bonnet-Delpon, 2006; Isanbor and O'Hagan, 2006; Yamazaki et al., 2009). In fact, ca. 25% of pharmaceuticals currently commercialised correspond to fluorinated compounds and from the ten most sold human pharmaceuticals in the year of 2015, four of them are fluorinated – Crestor® (rosuvastatin), Sovaldi® (sofosbuvir), Advair Diskus® (fluticasone propionate) and Januvia® (sitagliptin), respectively (Fig. 2) (Gilchrist, 2015; Murphy, 2016). Organofluorine pesticides such as fipronil, epoxiconazole and trifluralin, can also be found amongst the top best-selling agrochemicals in Europe and in the United States of America (Loi et al., 2011).

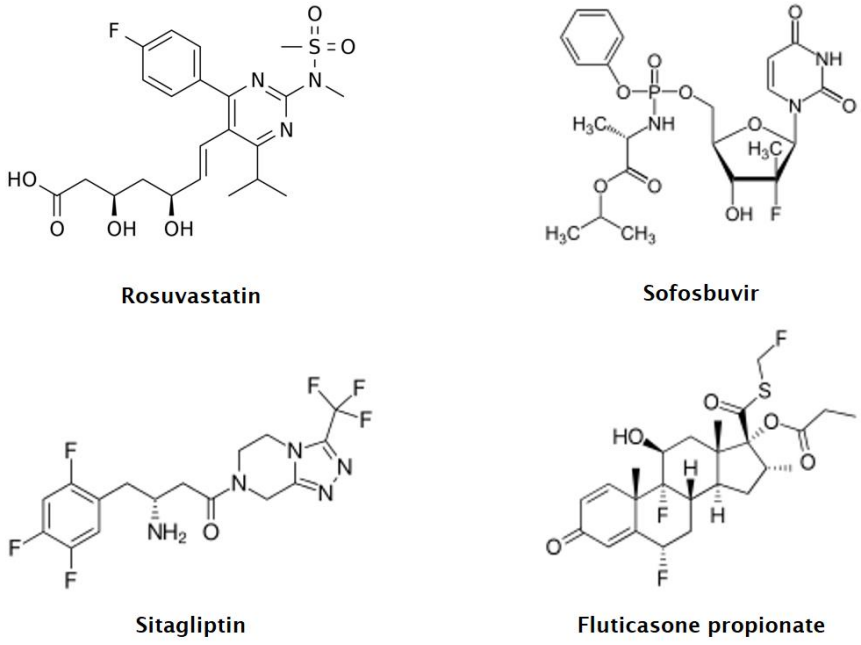


Figure 2. Chemical structure of the four top-selling fluorinated pharmaceuticals in 2015.

The reason why fluorine is becoming one of the most attractive heteroatoms in molecular design, is fully attributed to the atomic properties of this element which are transferred to the molecules it incorporates. In other words, its peculiar characteristics are mirrored in the compounds it incorporates, leading to the emergence of favourable properties.

One of the most significant attributes associated with the molecular incorporation of fluorine, is the increase in the metabolic stability of fluorinated structures (Zhang et al., 2012; Ojima, 2013). Due to their molecular strength and low reactivity, fluoroorganic compounds are likely to remain stable in blood circulation, reducing their susceptibility to detoxification mechanisms and also their potential for systemic toxicity (Ojima, 2013). On the other hand, the low likelihood of intermolecular interactions associated with their increased metabolic stability, promotes the selectivity of organofluorine compounds. By reducing their interaction with secondary targets, fluorinated molecules will exert their bioactivity more directly and efficiently.

Lipophilicity and membrane permeability is significantly promoted with the incorporation of fluorine in aromatic molecules, and thus fluorinated compounds have enhanced pharmacokinetics and pharmacodynamics properties (Zhang et al., 2012; Ojima, 2013). This is a favourable property for both pharmaceuticals and biocides, as it promotes their biological activity in biochemical and physiological targets.

As already referred, fluorine shares a similarity in steric size with hydrogen or a hydroxyl group (Jaccaud et al., 2000). This means that fluorine-substitution can generate congeners of desirable chemical structures with enhanced characteristics, without compromising its intended biological effect. The production of synthetic or semi-synthetic fluorinated compounds is becoming a common trend in medicinal chemistry, with a special focus on the biosynthesis of fluorinated analogues of natural products (Zhang et al., 2012).

2.4. Industrial significance of fluoroorganics

Fluorinated compounds hold several properties that are highly attractive for industrial purposes, where they may act as reagents, solvents, building blocks, polymers, among others. The physical, chemical and thermal stability of the C-F bond is the main reason why fluoroorganics are highly used in industry. The

distinctive solubility properties induced by molecular fluorination, make some organofluorine compounds to act as optimal industrial solvents, being compatible with most lipophilic substances or with other fluorinated compounds. These solvents are highly used in purification processes in fine-chemistry industries and in the synthesis of other fluoroorganic products (Spargo, 2005).

Fluorinated compounds may also serve as important building blocks, being frequently used as fluorine donors or precursors in the preparation of more complex organofluorines (Siegemund et al., 2000).

Fluorinated polymers have increased advantages, essentially associated with their high resistance, great isolating properties and non-permeability (Siegemund et al., 2000). As a result of their versatility, various fluoropolymers are currently being used in many applications, ranging from domestic appliances, cookware, textiles, clothing, medical equipment, or even in the formulation of firefighting foams (Siegemund et al., 2000). One good example of a widely used fluoropolymer is polytetrafluoroethylene, a waterproof and light polymer that is part of the famous materials Teflon® and GoreTex®.

3. Biodegradation and Bioremediation

Biodegradation is a biological process carried out by microorganisms that leads to the simplification of the molecular structure of a compound, as a result of the catabolic activity of microbial enzymes. Bioremediation, refers to the strategic employment of microorganisms with the capacity to attenuate a contamination scenario, either by removing or neutralizing the target contaminants (Crawford, 1998). Both these concepts are closely related, as bioremediation strategies rely on the biodegradation potential of microorganisms and make use of their capacities to ensure environmental restoration. During bioremediation, microbial removal or transformation of xenobiotics into less, or even non-hazardous products occurs (Karigar and Rao, 2011).

Since the 1980s, bioremediation processes have been employed for the remediation of oil spills and other hazardous products (Shannon and Unterman, 1993) and, more recently, they have also been applied in different contexts of contamination, mostly targeting micropollutants and emerging pollutants (Das and Dash, 2014).

Contaminants may also be removed from the environment through physicochemical processes, such as precipitation, coagulation, adsorption, biosorption or reverse osmosis (US-EPA, 2007; Wang and Chen, 2009; Das and Dash, 2014). However, when compared to these processes, bioremediation presents several advantages: (i) biological remediation of contaminated sites tends to be cheaper than physicochemical remediation techniques (Kumar et al., 2011); (ii) some physicochemical processes of remediation are highly invasive and, as a result, may yield secondary effects in the environment, (iii) bioremediation has the potential to mineralize the contaminants, i.e., to convert the contaminants into their constituent elements, as it is based on natural and recycling processes; (iv) bioremediation technologies are capable of removing environmental contaminants with minimal environmental impacts and, most of the times, without involving the transfer of contaminated waste or soil for *ex situ* treatment, as in many physicochemical processes (Kumar et al., 2011).

In spite of its several advantages, bioremediation also presents some limitations. Biological remediation strategies rely on the efficiency of metabolically-competent microorganisms, however these may not always be present or active at the bioremediation site. Also, metabolic reactions are always dependent on microbial viability, which in turn is highly influenced by variables that are hard to control in real-life scenarios, such as suitable environmental conditions or appropriate levels of nutrients (Kumar et al., 2011). Moreover, in order to ensure the expression of the key enzymes involved in the bioremediation processes, several conditions should be met, including adequate concentration of the target contaminant in the environment, suitable temperature, pH and redox conditions and suitable bioavailability of the contaminant. In addition, certain bioremediation strategies may affect the normal dynamics of ecosystems. For example, the use of non-autochthonous microbial species/microbial communities for bioremediation purposes may cause disturbances on the ecology and microbial dynamics of the indigenous microbiota of the site, impairing the natural functioning of that ecosystem (Thompson et al., 2005; Kumar et al., 2011).

3.1. Factors influencing biodegradation and bioremediation

Microbial metabolism is a central aspect of biodegradation and bioremediation, as it is determinant for the transformation and environmental removal of contaminants. Microorganisms are able to convert or even mineralize xenobiotics

through catabolic reactions, usually associated with energy consumption (Adams et al., 2015). These metabolic processes normally involve redox reactions and may be associated with respiration or other biological functions that are indispensable for cell viability and reproduction (Adams et al., 2015). Such reactions are highly influenced by various factors, either intrinsic to the microorganisms or associated with the environment where they are integrated, and will directly influence the overall effectiveness of biodegradation and, consequently, of the bioremediation strategy.

The capacity of microorganisms to transform, accumulate or mineralize contaminants is a fundamental part of bioremediation (Karigar and Rao, 2011). In a contamination scenario, prior knowledge on this aspect is needed for the outlining of an efficient bioremediation strategy. The capacity of microbial cells to utilize xenobiotic structures as sources of energy is not a common phenotype, since their catabolic enzymes did not have a natural evolution process with these compounds. While microbial metabolism can be extremely versatile, some contaminants remain recalcitrant to biological degradation, especially when having high molecular weights and bearing complex ring structures and halogen substituents (Das and Dash, 2014). Many xenobiotic compounds are biodegraded only through cometabolic processes. Cometabolism can be defined as a metabolic interactive effect between two substrates, where usually one is actively metabolised, being used as a source of carbon and/or energy, and the other one is unable to support microbial growth (Criddle, 1993). Different variations of cometabolic reactions might occur in the environment, contributing to the conversion of various chemical compounds, either by supporting an increase in microbial density or by improving metabolic performances (Dean-Ross et al., 2002).

The capacity to degrade a certain contaminant or group of contaminants may be intrinsic to autochthonous microbial species/microbial communities or not. If the native microbiota of a contaminated site includes microorganisms capable of metabolizing a contaminant, then the bioremediation strategy can make use of these microorganisms to remove the pollutants (Das and Dash, 2014). There are cases though, where the introduction of exogenous, non-native, microbial species is needed in order to remove specific contaminants (bioaugmentation). In any of these situations, the microorganisms responsible for the biodegradation processes must be able to reach their optimal activity and metabolic peak in contaminated sites, so that they are able to remove or neutralize the xenobiotics. In order to

achieve this, in some cases it is necessary to add nutrients, essentially nitrogen and phosphorous, to the contaminated site (biostimulation).

While knowledge on microbial metabolic potential is a key factor in bioremediation processes, information on other variables, such as environmental factors or contamination dynamics is also very important. Environmental factors include a wide array of physical, chemical and biological conditions that confer additional complexity to the whole bioremediation process, as they influence both microbial activity and the environmental dynamics of the contaminants. Among the broad spectrum of environmental variables the most relevant ones are: geophysical characteristics of the affected site, nutrient availability, presence of oxygen (or other electron acceptors), temperature and pH (Das and Dash, 2014; Adams et al., 2015). Site characteristics should be properly explored prior to the implementation of a bioremediation strategy. Besides influencing the distribution and bioavailability of the contaminants, it will also determine microbial survival rate by modulating oxygen content, nutrient availability, water content, among other factors (Adams et al., 2015). Nutrients are essential elements for the survival, viability and multiplication of microbial cells, with carbon, hydrogen and nitrogen being needed in greater quantities over other elements (Das and Dash, 2014). Temperature, pH or oxygen content, are vital factors for microbial survival and their optimal levels will depend significantly on the type of microorganisms involved in the biodegradation mechanisms.

Regarding the dynamics of contamination, the magnitude, extent, mobility and toxic potential of the involved contaminants are essential aspects (Das and Dash, 2014). Knowledge on this will allow to clarify the hazardous nature of the contamination and to more properly define the bioremediation strategy. The characteristics of the contamination may pose as a limiting factor in bioremediation – the type of contaminants, as well as their formulation, concentration and bioavailability will always determine the likelihood of microbial degradation and, thus, the efficacy of the bioremediation process (Adams et al., 2015). Geological and soil characteristics of the site are also important in the environmental dynamics of contaminants, as they influence their mobility, distribution and bioavailability.

4. Microbial transformation and degradation of fluoroorganic compounds

The recalcitrant nature of fluoroorganics is widely acknowledged and has been verified for various fluorinated compounds (Key et al., 1997; Neilson and Allard, 2002). Yet, when concerning the biodegradation of these compounds, scientific research has focused more on fluoroaromatic structures, with this topic being less explored for aliphatic organofluorines.

The physicochemical properties of these compounds are an important reason behind their resistance to microbial catabolism and can be almost fully attributed to the significant negative inductive effect associated with fluorine's high electronegativity. This creates a stereochemical and electronic unbalance on the whole molecular structure, generally preventing the electrophilic attack of molecular oxygen, which constitutes a primary step in most aerobic metabolic pathways (Kiel and Engesser, 2015). Moreover, some fluoroorganics can act as enzymatic inhibitors, being capable of irreversibly inhibiting enzymatic activity (Neilson and Allard, 2002). Thus, their recalcitrance may also be due to their capacity of inactivating their potential biocatalysts, preventing their biotransformation. As a result of these characteristics, the biochemical interaction between organofluorine compounds and microorganisms often results in their incomplete degradation or no degradation at all (Neilson and Allard, 2002). Complete defluorination of a fluoroorganic usually leads to its mineralization, especially if occurring as a primary step on the catabolic pathway, since elimination of fluoride is a critical step in the biodegradation of fluorinated compounds (Kiel and Engesser, 2015). This is particularly relevant when fluorination occurs in core structures of aromatic organofluorines or in short-chained aliphatic compounds, as in both these cases fluorine's inductive effects are more evident throughout the whole molecular structure (Kiel and Engesser, 2015).

As defluorination capacity is a characteristic not commonly present in most microorganisms, fluoroorganic compounds are more likely to be biodegraded through unspecific reactions, as those observed in cometabolic pathways. Yet, even in these conditions, fluorinated compounds might not be fully metabolized (Kiel and Engesser, 2015). The concomitant presence of a fluorinated compound and a co-substrate, might induce cometabolic reactions due to structure similarity or to growth stimulation of the microbial population (Kiel and Engesser, 2015). The first situation requires two substrates to be structurally related, and occurs when the

presence of the growth substrate is capable of inducing enzymes able to catalyse the breakdown of its recalcitrant analogue, while the second situation corresponds to the use of substrates capable of supporting microbial growth, leading to an increase of catabolic enzymes and, consequently improving the chances of degradation of the fluorinated substrate.

Aliphatic and aromatic organofluorines have distinct stereochemical and biochemical demands when concerning their biological transformation, thus exhibiting different pathways through which they may be metabolized. Aliphatic fluoroorganics are generally smaller and chemically simpler than fluorinated aromatics, with the exception of perfluorinated aliphatics that bear additional functional groups or several ring structures that may influence their biodegradability. As a result of their simpler structures, defluorinating reactions are common primary steps in the microbial degradation of aliphatics, and some different enzymes have been reported to catalyse such reactions (Fetzner and Lingens, 1994). Concerning aromatic structures, it has been shown that their biodegradation share some similarities with the degradation of their non-fluorinated analogues, such as in the case of several phenols, benzenes, benzoates and anilines, whose metabolic pathways are well established (Boersma et al., 2001; Carvalho et al., 2006; Iwai et al., 2009). In these compounds, fluoride ion removal is an essential step in their degradation because it facilitates the consequent transformation of the resulting substrate and avoids the generation of unwanted, dead-end metabolites, which may be more persistent or toxic than the parental compound (Kiel and Engesser, 2015). Defluorination may occur before or after fission of the aromatic ring, but may be hindered depending on the position where the fluorine atom is on the aromatic ring or if there is more than one ring structure, as in the case of polycyclic compounds (Neilson and Allard, 2002; Murphy et al., 2009).

Genetic mechanisms are also an important factor in the microbial degradation and transformation of fluoroorganic compounds. Gene transfer can, in some cases, lead to the emergence of novel defluorinating pathways or endow non-metabolically competent microorganisms with suitable catabolic mechanisms to attack fluorinated molecules. The acquisition of such genotypes may be the result of horizontal gene transfer or though the integration of functional replicons, mediated by integrase enzymes (Janssen et al., 2001). Adaptation processes can also be a way through which microorganisms acquire capacities to transform and

defluorinate fluoroorganic molecules, and gene transfer has an important influence in such processes. Additionally, environmentally-driven genetic mutations, such as recombinations, are also relevant in microbial enrichment and constitute important adaptation processes to fluorinated xenobiotics (Janssen et al., 2001).

5. Genomic and metagenomic approaches in biodegradation studies

In biodegradation studies it is very important to properly identify the metabolically-competent microorganisms as well as to understand their microbial dynamics.

Much of this valuable information is now more easily accessible, thanks to the the development of omics tools. More specifically, genomic and metagenomic approaches have allowed to deepen the investigation of the microbial world, allowing a clearer identification of microbial species, and contributing to the understanding of microbial community dynamics, also visualizing segments of the microbiome (essentially uncultured microorganisms) which were invisible otherwise.

Genomics corresponds to the study of gene function and structure, allowing mapping and elucidating biological systems and reactions. In microbiology, genomics revolutionized the taxonomy and phylogeny of microbial species through the analysis of specific genes with taxonomic value. In bacteria, the 16S ribosomal RNA gene (16S rRNA gene) has been widely used for the phylogenetic identification of bacterial isolates as it is highly conserved within bacterial species, showing only some variable regions (Coenye and Vandamme, 2003). Prior to 16S rRNA gene sequence analysis, bacterial taxonomy was based on morphological, biochemical and physiological characteristics of microbial strains, which was often a subjective procedure (Handelsman, 2004). In biodegradation/bioremediation studies, 16S rRNA gene sequencing allowed to more accurately identify bacterial species with biodegradation capacities. A myriad of microorganisms capable of remediating and neutralizing numerous environmental contaminants have been identified thanks to this genomic-based approach.

Metagenomics is the application of genomic-based principles for the analysis of microbial communities directly derived from environmental samples. One major advantage of this tool, when compared with other genomic approaches, is that it enables the combined analysis of cultured and non-cultured microorganisms,

allowing understanding the microbial composition within a whole community. Through this approach, it is possible to obtain high-resolution genetic information of complex microbial systems, such as community shifts and dynamics and microbial composition, diversity and structure (Bell et al., 2013). Therefore, this type of approach allows better understanding how a microbial community responds and adapts to the presence of a target contaminant (Bell et al., 2013). For example, microbial diversity has been regarded as a good indicator of ecosystem function with environmental microbiomes with high levels of microbial diversity being usually more resistant to anthropogenic disturbances (Bissett et al., 2007; Allison and Martiny, 2008). Metagenomic approaches allow understanding how environmental microbiomes are affected by the presence of contaminants, which is very important for the assessment of the environmental impact caused by these compounds.

6. Aim and outline of this thesis

The utilization of fluoroorganic compounds is increasing worldwide, accompanied by a proportional increment on their environmental presence and distribution. Due to the fact that the majority of these compounds are emergent pollutants, a lot is yet to be known regarding their biodegradability and hazardous nature. Consequently, knowledge on the biodegradability of these compounds and on suitable bioremediation technologies capable of mitigating the environmental impact of fluorinated xenobiotics is urgently needed. In this context, the work developed in this master dissertation focused in the investigation of the biodegradation of fluoroorganic compounds with different structures (aliphatics and aromatics) and applications.

The present thesis is structured as follows: in Chapter 1, a general introduction is provided, presenting the state of the art concerning the properties, applications and biodegradation of fluoroorganic compounds and also outlining key concepts and definitions associated with biodegradation and bioremediation of xenobiotics; the experimental approach contemplated in this master thesis is presented in Chapters 2 and 3. In Chapter 2, the biodegradation of structurally related aliphatic carboxylic fluoroorganics with many industrial applications is explored, while in Chapter 3, the biodegradation of an aromatic structure, a widely used veterinary fluoroquinolone, when present individually and in mixture with a second antibiotic,

a veterinary cephalosporin, is investigated. In Chapter 4, some final remarks are presented, including a general discussion on both experimental works and main conclusions.

Both experimental works integrated in this thesis were submitted to international peer-reviewed scientific journals, with the following references:

1. **Alexandrino DAM**, Mucha AP, Almeida CMR, Gao W, Jia Z and Carvalho MF. (2016). *Biodegradation of the veterinary antibiotics enrofloxacin and ceftiofur and associated effects on microbial community dynamics.* Submitted to SCIENCE OF THE TOTAL ENVIRONMENT.

2. **Alexandrino DAM**, Ribeiro I, Pinto LM, Cambra R, Oliveira RS, Pereira F and Carvalho MF. (2016). *Biodegradation of mono-, di- and trifluoroacetate by microbial cultures with diferente origins*. Submitted to NEW BIOTECHNOLOGY.

CHAPTER 2

BIODEGRADATION OF MONO-, DI- AND TRIFLUOROACETATE BY MICROBIAL INOCULA WITH DIFFERENT ORIGINS

(submitted to NEW BIOTECHNOLOGY)

1. Introduction

Due to the useful properties that fluorine confers to organic molecules, the use of synthetic organofluorines for industrial, medical and agricultural applications has been significantly increasing in the last decades (Kiel and Engesser, 2015). As a result of their vast applications, fluoroorganic molecules are becoming pollutants of several environmental compartments, where they may persist for long periods of time due to the recalcitrant nature of many of these molecules (Banks et al., 1994; Thayer, 2006). The degradation of organofluorine compounds constitutes a challenge to microorganisms not only because the environmental pollution originated by these compounds is a relatively recent problem, causing microorganisms to be exposed to compounds so far unknown, but also because the C-F bond of organofluorines has one of the highest known energies, making it challenging to cleave (O'Hagan, 2008).

Fluoroacetates (FAs) are a family of carboxylic aliphatic organofluorines composed by mono- (MFA), di- (DFA) and trifluoroacetate (TFA) that are highly soluble in water, non-volatile and, as a result, likely to be mobile in the environment. MFA is a naturally-occurring organofluorine and its synthetic form is used in some countries as a vertebrate pesticide. This compound is highly toxic, especially to mammals, where it acts as a potent inhibitor of the tricarboxylic acid cycle (O'Halloran et al., 2005; Camboim et al., 2012). A number of tropical and sub-tropical plants are capable of producing and accumulating MFA, using it as a defence mechanism against herbivores (Marais, 1944; O'Hagan et al., 1993; Davis et al., 2012), and a few *Streptomyces* species have also been found to produce it (Sanada et al., 1986; Deng et al., 2014). MFA is also an important building block and an intermediary reagent used in the industrial synthesis of several fluorinated antibiotics and synthetic aminoacids and is a secondary product resultant from the microbial metabolism of several fluorinated pharmaceuticals and industrial reagents (Ihara et al., 1996; Percy, 1997; Goncharov et al., 2006). DFA is used in the chemical synthesis of various fluorinated compounds and is produced during the microbial metabolism of a range of organofluorines (Fox et al., 1990; Visscher et al., 1994; Ihara et al., 1996; Percy, 1997; Morii et al., 2004; Ge et al., 2007). This compound is suggested to result from the thermolysis of several commercial fluorinated polymers (Ellis et al., 2001). TFA is an important derivative of the tropospheric degradation of several HCFCs and HFCs, and is also a resulting product from the abiotic breakdown of fluorinated polymers (Martin et al., 2000; Ellis et al., 2002).

In addition, this compound is widely used as a building block for the production of various synthetic fluoroorganic compounds (Tamura et al., 1993; Linderman et al., 1994; Boivin et al., 1995).

FAs have been reported to occur in several environmental compartments, being the aquatic media their major environmental sink (Wang et al., 2004). TFA has been detected in seasonal wetlands, marine environments, rainwater and lotic environments, in concentrations ranging from 30 to 600 ng L^{-1} (Cahill and Seiber, 2000; Cahill et al., 2001; Römpp et al., 2001; Frank et al., 2002; Scott et al., 2005). Although current environmental concentrations of TFA appear to be non-toxic to microorganisms and animals, presenting only mild toxicity to some plants, its recalcitrance may eventually lead to the accumulation of higher concentrations, thus increasing the potential for ecosystem damage (Berends et al., 1999; Bott and Standley, 1999; Smit et al., 2009). The environmental occurrence of MFA is mainly linked with its use as a pesticide, that is applied aerially or in baits, though releases through discharges of chemical industries may also occur (Ogilvie et al., 2010). The physicochemical properties of MFA (water solubility, lack of volatility and low Kow) suggest considerable mobility in the environment, being likely to reach groundwater streams and even surface waters. The environmental dynamics of DFA remain poorly explored in the literature but its structural similarity to the other FAs, namely regarding its physicochemical properties, suggests a similar environmental behaviour.

MFA was found to be biodegraded by different soil microorganisms (Gentle and Cother, 2014). Kelly (1965) reported for the first time the bacterial degradation of MFA, and other MFA-degrading bacteria have been isolated afterwards (Meyer et al., 1990; Emptage et al., 1997; Davis et al., 2012). Microbial degradation of this compound is usually mediated by the enzyme fluoroacetate dehalogenase, which catalyses the cleavage of the C-F bond in the molecule, yielding glycolate (Goldman, 1965; Kawasaki et al., 1992; Kurihara et al., 2000). Biodegradation of TFA has been reported to occur under anaerobic conditions, though its aerobic conversion to fluoroform has also been described (Visscher et al., 1994; Kim et al., 2000). However, current results on TFA biodegradation lack reproducibility and, thus, more studies are needed. DFA has been identified as a secondary metabolite resultant from the anaerobic biodegradation of TFA, being further converted into MFA and then acetate (Visscher et al., 1994). Though this data suggests degradation of DFA under anaerobic conditions, to the best of our knowledge no

studies on the aerobic biodegradation of this compound are available in the literature. Moreover, as these compounds may occur simultaneously in the environment, it is important to understand how the degradation of each compound is affected by the presence of its analogues. In this context, our work aimed to investigate the aerobic biodegradation of MFA, DFA and TFA as sole carbon sources and in mixtures of two FAs. In addition, co-metabolic degradation of DFA and TFA in the presence of their non-fluorinated analogue, acetate, was also studied. Biodegradation was investigated using microbial inocula from different origins.

2. Materials and Methods

2.1. Microbial inocula

Sediment and rhizosphere samples of *Phragmites australis (Cav.) Trin. ex Steud*. were collected from a site in Estarreja, Portugal with a long history of industrial chemical contamination (Oliveira et al., 2001), and used as an environmental source of microorganisms. An activated sludge consortium originated from a municipal wastewater treatment plant (Gondomar, Porto) was also used as inoculum for this study. This inoculum was obtained by centrifuging 40 mL of activated sludge (5000 rpm for 15 min at 4 ºC), washing twice the resultant pellet with a minimal salts medium (MM) and resuspending it in the same medium to one tenth of its original volume.

2.2. Biodegradation experiments

Biodegradation experiments were performed in batch mode in 250 mL flasks with 70 mL of sterile MM. MM contained (per litre of ultra-pure water): $Na_2HPO_4 \cdot 2H_2O$ 2.7 g, KH_2PO_4 1.4 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g and 10 mL of a trace elements solution with the following composition, per litre: $Na_2EDTA \cdot 2H_2O$ 12.0 g, NaOH 2.0 g, $MnSO_4 \cdot 4H_2O$ 0.4 g, $ZnSO_4 \cdot 7H_2O$ 0.4 g, H_2SO_4 0.5 mL, Na_2SO_4 10.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 2.0 g, $CuSO_4 \cdot 5H_2O$ 0.1 g and $CaCl_2$ 1.0 g. Flasks were inoculated with 5 g of fresh sediment or rhizosphere samples and for the activated slugge consortium, flasks were inoculated in order to have an initial optical density (OD) at 600 nm of 0.1. Cultures were fed with FAs individually, in mixtures of two FAs and, for DFA and TFA, in cometabolism with acetate. When fed

individually, FAs were supplemented at a concentration of 20 mg L^{-1} (0.20, 0.17 and 0.15 mM for MFA, DFA and TFA, respectively), while in the binary mixtures of FAs, each compound was fed at the concentration of 10 mg L^{-1} (0.10, 0.085 and 0.074 mM for MFA, DFA and TFA, respectively). Cultures in cometabolism with acetate were supplemented with DFA or TFA at the concentration of 5 mg L^{-1} (0.042 and 0.037 mM for DFA and TFA, respectively) and fed three times a week with 500 mg L^{-1} of sodium acetate. In the latter treatment, cultures were weekly transferred to new sterilised flasks in order to ensure sufficient oxygen for the aerobic degradation of the target compounds. Biodegradation of FAs was followed during a three week period, after which half of the cultures were transferred to new flasks containing the same proportion of MM and re-fed with the respective carbon sources. Cultures were incubated under aerobic conditions, in a rotary shaker (130 rpm) at 25 ºC in the dark. Abiotic controls consisting in MM supplemented individually with each of the FAs (5 mg L^{-1}) and incubated under the same conditions were also included. Experiments were conducted in duplicate. FAs biodegradation was followed by periodically measuring bacterial growth and fluoride ion release.

2.3. Bacterial characterization of MFA-degrading cultures

The bacterial composition of MFA degrading cultures was analysed by spreading several tenfold dilutions of culture samples onto minimal salts agar plates supplemented with MFA as sole carbon source and Plate-Count Agar (PCA). The plates were incubated at 25 ºC until growth was detected. Bacterial composition was analysed by visual inspection and morphologically distinct colonies were purified by streaking the different colonies in new agar plates.

2.4. Biodegradation capacity of bacterial isolates obtained from MFA-degrading cultures

The capacity of the different bacterial strains isolated from the MFA-degrading cultures to degrade this compound in axenic cultures was investigated by inoculating single strains into 30 mL sterile flasks, filled to two thirds of their volume with MM and supplemented with MFA at 20 mg L^{-1}. The initial OD (600 nm) of the cultures was 0.1. Flasks were incubated in a rotary shaker (130 rpm at 25 ºC), in the dark. Biodegradation was followed along a three week period by monitoring bacterial growth and fluoride ion release.

A bacterial culture consisting of a mixture of all MFA-degrading isolates was also created and used as inoculum for investigating its capacity to degrade DFA and TFA, fed individually as sole carbon source (20 mg L^{-1}) and in cometabolism with MFA (20 mg L^{-1} of MFA and 5 mg L^{-1} of DFA or TFA).

2.5. Identification of MFA-degrading isolates

All the isolates capable of degrading MFA as single strains were identified through 16S rRNA gene sequence analysis. DNA was extracted from colonies obtained from minimal salts agar plates supplemented with MFA or PCA plates, following a standard phenol-chloroform extraction method, as described elsewhere (Sambrook et al., 1989). Briefly, bacterial colonies were transferred to 1.5 mL microtubes to which STE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 8.0), sodium dodecyl sulphate (20%) and proteinase K (20 mg mL^{-1}) were added. The mixture was incubated overnight at 56 ºC with gentle shaking. After the incubation period, samples were transferred to Light Phase Lock Gel tubes to which phenol:chloroform:isoamyl (25:24:1) and chloroform:isoamyl (24:1) alcohols were sequentially added, after intercalated centrifugations (14000 rpm for 3 minutes) to ensure the separation of the aqueous and organic phases. Finally, the obtained DNA was concentrated through ethanol precipitation and the resulting pellets were dried under sterile conditions at room temperature. DNA extracts were then dissolved in 50 µL of sterilised water.

Extracted DNA was amplified by Polymerase Chain Reaction (PCR) using the universal primers 27F and 1492R (Weisburg et al., 1991). PCR reaction mixture contained 2 µM of the universal primers, a Multiplex PCR Master Mix (Qiagen, Valencia, CA) and template DNA sample. Negative controls were included and consisted on the same PCR reaction mixture in which DNA was replaced by DNase, RNase and protease-free water (5 Prime). PCR amplification conditions included initial denaturation at 95 ºC for 15 minutes, followed by 30 cycles at 94 ºC for 30 seconds, 48 ºC for 90 seconds (annealing step) and 72 ºC for 2 minutes, and a final extension at 72 ºC for 10 minutes. Amplification products were separated by electrophoresis in a 1.5% agarose gel containing SYBR® Safe (ThermoFisher Scientific, Massachusetts, USA) at 150 V for 30 minutes. DNA fragments were visualised under UV light in a BioRad Molecular Imager® Gel Doc™ XR+ with Image Lab™ Software and those showing amplification bands with a suitable size

(~1500bp) were sent for sequencing at i3S – Instituto de Investigação e Inovação em Saúde (Porto, Portugal).

2.6. Analytical methods

Fluoride release was analysed by potentiometry, through the measurement of the concentration of fluoride ion in the supernatant of culture samples, using a fluoride-selective electrode (Crison 9655 C, Crison Instruments, S.A., Barcelona, Spain). Prior to sample analysis, a calibration curve was constructed using standards of sodium fluoride (0.001 to 1 mM) prepared in MM. A total ionic strength adjustment buffer (TISAB III) was supplemented to the samples and standards in a 1:10 ratio.

Microbial growth was monitored through the measurement in a spectrophotometer (Model V-1200, VWR International, LLC, Pennsylvania, USA) of the optical density (OD) at 600 nm of culture samples.

3. Results

3.1. Biodegradation of FAs by the different microbial inocula

The microbial capacity to degrade three structurally related FAs, MFA, DFA and TFA, as sole carbon sources, in mixtures of two FAs and in cometabolism with acetate, was investigated using microbial inocula with distinct origins. Fluoride release was used as a key biodegradation indicator, since the main obstacle to the microbial degradation of these compounds lies in the presence of this atom in their molecular structures.

When supplemented as a sole carbon source, only MFA was degraded by the tested microbial inocula. Activated sludge consortium showed complete defluorination of MFA since the beginning of the experiment, whereas the treatments inoculated with sediment or rhizosphere samples revealed a gradual increase in MFA degradation performance (Fig. 3). In these latter cultures, total defluorination was also achieved: for cultures inoculated with sediment samples this was obtained when fed a second time with MFA, while for rhizosphere cultures this was observed in the following feeding period (Fig. 3). Total defluorination of MFA was maintained in further MFA

feedings for an additional period of 2 months. None of the tested microbial inocula were capable of defluorinating DFA or TFA, either when supplemented as sole carbon sources or in cometabolism with acetate.

Biodegradation of MFA in mixture with DFA or TFA was also investigated. A mixture of DFA and TFA was not considered since no biodegradation had been obtained when these compounds were supplemented individually. When MFA was supplemented with DFA, only a small fraction of fluoride was detected in the culture medium of the different tested microbial consortia (Fig. 4).

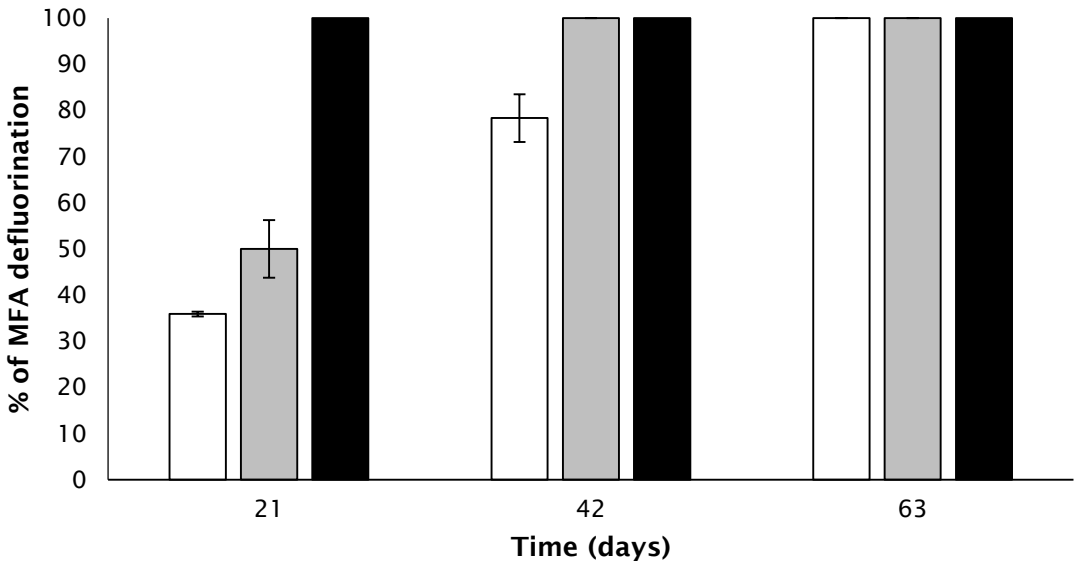


Figure 3. Biodegradation performances, based on fluoride release, of MFA supplemented as a sole carbon source during a two months period. White bars represent rhizosphere inoculum, grey bars, sediment inoculum and black bars, activated sludge consortia. Days 21, 42 and 63 correspond to the end of the 1st, 2nd and 3rd MFA feeding periods, respectively. The results represent the mean of duplicates and error bars show standard deviation.

The low concentration of released fluoride in these cultures indicates that the simultaneous presence of the two FAs not only did not stimulate the biodegradation of DFA, but also produced a negative effect in the biodegradation of MFA, as the obtained fluoride concentration was not proportional to the complete defluorination of this compound (Fig. 4). In the cultures supplemented with a mixture of MFA and TFA, the concentration of fluoride ion analysed in the culture medium was higher than that obtained in the cultures fed with MFA and DFA, and the extent of fluoride released suggests that MFA was fully degraded as

it is in agreement with its stoichiometric defluorination. This result suggests that, unlike DFA, the presence of TFA in the mixture does not interfere with MFA biodegradation and that, similarly to what happened with DFA, the addition of MFA does not stimulate biodegradation of TFA. In the cultures fed with MFA, both as sole carbon source or in mixture with TFA, a slight OD increase was observed (data not shown), though for cultures inoculated with sediment or rhizosphere samples this parameter could not be analysed along the first three feeding periods due to the interference of the inocula in this analysis.

Abiotic controls were also established and followed in parallel with the biodegradation experiments, revealing no fluoride release in any of the flasks under the tested experimental conditions.

3.2. Characterization of MFA-degrading bacterial consortia and biodegradation capacity of the isolated strains

All the cultures degrading MFA (individually or in mixture with TFA) were analysed in terms of their bacterial diversity. A total of 43 bacterial isolates were obtained from the degrading cultures: 12 strains were recovered from activated sludge, 15 strains from cultures inoculated with rhizosphere samples and 16 strains from cultures inoculated with sediment samples (Table 2). All these isolates were tested individually for their capacity to degrade MFA when supplemented as a sole carbon source, revealing that out of the 43 isolates recovered, 15 were capable of completely defluorinating MFA (Table 2). The highest number of MFA-degrading isolates was obtained from activated sludge consortia.

A mixed culture composed by all MFA-degrading isolates was also established and tested for its capacity to degrade DFA and TFA as sole carbon sources and in cometabolism with MFA. Based on fluoride release, no biodegradation of DFA and TFA, fed individually, was observed with this consortium. When MFA was supplemented as a co-metabolite, the results obtained were very similar to the ones previously observed with the mixtures of two FAs, i.e., the concentration of fluoride ion analysed in the culture medium when MFA was fed with TFA correlated with the total defluorination of MFA, suggesting that this defluorination pattern is attributed solely to the degradation of MFA, but when DFA was present in the mixture, biodegradation of MFA was inhibited and only ca. 10% of this compound was defluorinated (Fig. 5).

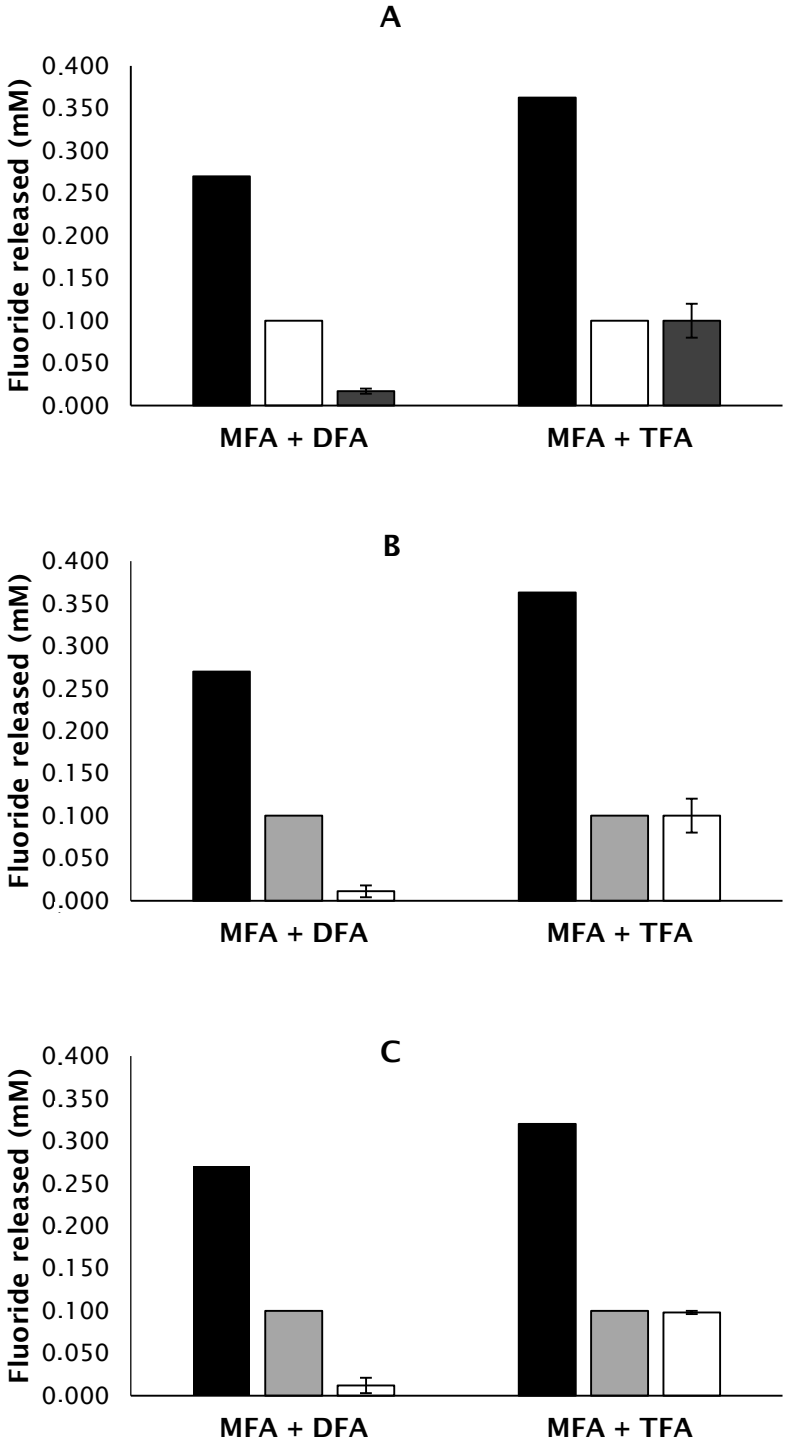


Figure 4. Defluorination performance of the tested microbial consortia when supplemented with mixtures of FAs after two feeding periods. **A** – activated sludge consortia; **B** – rhizosphere inoculum; **C** – sediment inoculum. Black bars show expected fluoride concentrations considering complete defluorination of both FAs in the mixture, grey bars show expected fluoride concentration considering total defluorination of only MFA (represented as the molarity of the stoichiometric release of the fluoride anion) and white bars show the concentration of fluoride ion released to the culture medium. Results represent the mean of duplicates and error bars are relative to standard deviation.

These results suggest that the culture consisting of the mixture of all MFA-degrading isolates was unable to metabolise DFA and TFA, being capable of defluorinating MFA in the presence of TFA, but not in mixture with DFA.

3.3. Identification of the MFA-degrading bacterial isolates

Bacterial isolates capable of degrading MFA as sole carbon source were identified through 16S rRNA gene sequence analysis. The isolates were identified as 9 distinct species, belonging to different genera, mainly assigned to the *Proteobacteria* phylum (Table 2). Activated sludge comprised MFA-degrading isolates belonging to 6 genera: *Stenotrophomonas*, *Herbaspirillum*, *Delftia*, *Pseudomonas*, *Comamonas* and *Achromobacter*. The genus *Pseudomonas*, as well as the species *Comamonas testosteroni* and *Achromobacter anxifer* were present in both activated sludge and cultures inoculated with sediment samples. An isolate belonging to the genus *Chryseobacterium* were also obtained from these latter cultures. In the cultures inoculated with rhizosphere samples, isolates capable of degrading MFA as single strains were found to belong to *Variovorax*, *Arthrobacter* and *Pseudomonas* (Table 2).

4. Discussion

Aliphatic organofluorines represent a class of compounds usually regarded as common environmental pollutants (Neilson and Allard, 2002). The critical step in the biodegradation of these compounds is the removal of fluoride ion (Kiel and Engesser, 2015). Complete defluorination of FAs was reported to yield easily degradable compounds that may be readily dissipated from the environment and have no potential for ecosystems damage, such as glycolate, a known secondary product of the biodegradation of MFA, or acetate, which is thought to result from the anaerobic biodegradation of TFA (Visscher et al., 1994; Kurihara et al., 2000).

Table 2. Microbial strains isolated from the different MFA-degrading consortia and taxonomic identification of the microbial isolates capable of degrading MFA as sole carbon source

Inoculum	Carbon source supplemented to the medium	Number of microbial isolates recovered	Number of isolates with capacity to degrade MFA	Identification of MFA degrading microorganisms	GenBank accession numbers
Activated sludge	MFA	5	3	*Comamonas testosteroni* strain MFA1	**KX400799**
				Stenotrophomonas maltophili strain MFA2	**KX400881**
				Herbaspirillum frisingense strain MFA4	**KX756676**
	MFA and TFA	7	3	*Delftia acidovorans* strain MFA5	**KX400852**
				Pseudomonas putida strain MFA15	**KX400880**
				Achromobacter anxifer strain MFA16	**KX398363**
Rhizosphere	MFA	8	1	*Pseudomonas sp.* strain MFA9	**KX404994**
	MFA and TFA	7	2	*Variovorax paradoxus* strain MFA10	**KX400967**
				Arthrobacter humicola strain MFA12	**KX400776**
Sediment	MFA	8	1	*Chryseobacterium taeanense* strain MFA25	**KX400798**
	MFA and TFA	8	3	*Achromobacter anxifer* strain MFA31	**KX400775**
				Pseudomonas sp. strain MFA32	**KX756677**
				Comamonas testosteroni strain MFA35	**KX400851**

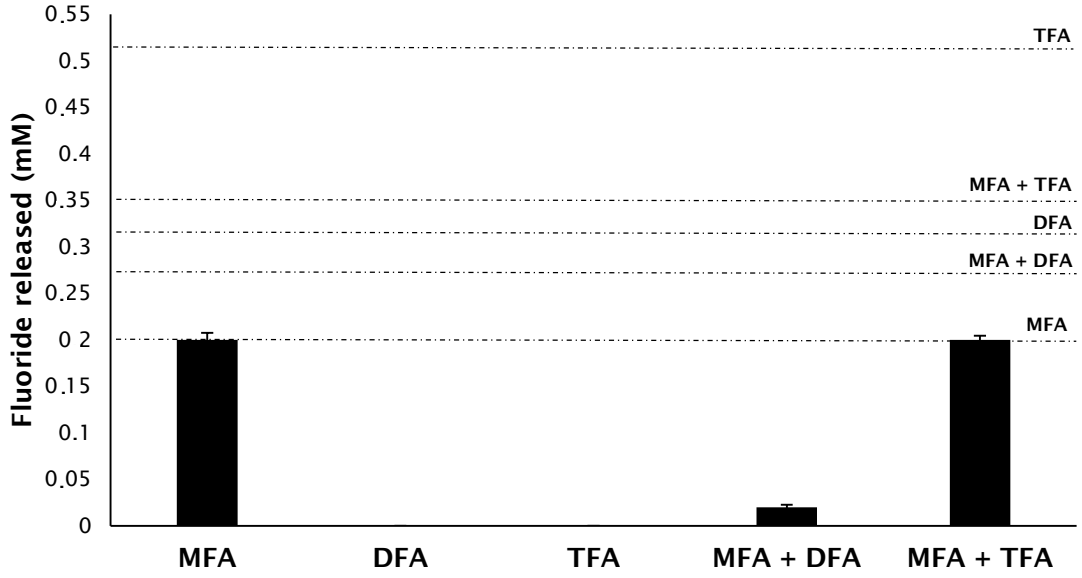


Figure 5. Biodegradation based on fluoride release of FAs supplemented as sole carbon sources and in cometabolism with MFA, by a mix of the 13 MFA-degrading microbial isolates. Dotted lines indicate theoretical fluoride concentration corresponding to complete defluorination of the tested compounds (represented as the molarity of the stoichiometric release of the fluoride anion). Results represent the mean of duplicates and error bars show standard deviation.

Complete defluorination of MFA supplemented as a sole carbon source was achieved in the cultures inoculated with activated sludge, rhizosphere and sediment samples. While activated sludge cultures readily defluorinated the supplemented MFA, an acclimation period was necessary for the other two microbial consortia to degrade the target compound. This may be due to activated sludge cells being in a more active metabolic condition than the other two microbial consortia, as these communities are typically subjected to high organic loads, having an easy access to growth substrates, and to high selective pressures, essentially due to the presence of a wide range of organic molecules in wastewaters. Nonetheless, the fact that the other tested microbial inocula also degraded MFA, indicates that microorganisms capable of metabolising this compound were originally present in these microbial consortia, though they needed an acclimation period in order to prevail in the communities. Due to the fact that no fluoride release was obtained in abiotic controls containing MFA, defluorination observed in the tested cultures can be solely attributed to the biological action of microorganisms in these cultures. Biodegradation of MFA has been reported before (Meyer et al., 1990; Wong et al., 1992; Camboim et al., 2012; Davis et al., 2012; Gentle and Cother, 2014). Most of the described MFA-degrading microorganisms originated from environments where MFA was known to be

present, such as soils in the neighbourhood of MFA-producing plants or soils adjacent to baits impregnated with this compound (Meyer et al., 1990; Gentle and Cother, 2014). However, isolation of MFA-degrading microorganisms has also been reported from samples not contaminated with this compound, indicating that the microbial capacity to metabolise MFA is widespread in the environment and among microorganisms (Wong et al., 1992; Camboim et al., 2012; Davis et al., 2012). This is in agreement with our results, as the obtained MFA-degrading isolates originated from environmental samples where MFA is not expected to be present.

DFA and TFA were not defluorinated by any of the tested cultures along an enrichment period of ca. 4 months. The absence of TFA defluorination under aerobic conditions is in agreement with the results reported by other authors, while DFA biodegradation has never been investigated to the best of our knowledge (Visscher et al., 1994; Benesch et al., 2002). Visscher et al. (1994) reported the accumulation of a dead-end metabolite, identified as fluoroform, resultant from the aerobic biodegradation of TFA. This metabolite still holds the trifluoromethyl group in its structure, and is more toxic than the parent compound. Benesch et al. (2002) found no aerobic biodegradation of TFA along a three month period by microbial communities from vernal pool soils. Contrastingly, complete defluorination of TFA under anaerobic conditions has been reported by Visscher et al. (1994) and Kim et al. (2000), with TFA (in concentrations ranging from 0.2 to 51 mg L^{-1}) being reductively dehalogenated under methanogenic conditions to DFA, MFA and acetate. Co-supplementation of the microbial cultures with acetate, a compound structurally similar to FAs and a common microbial substrate, did not produce a positive effect in defluorination of DFA or TFA. The co-feeding of substrates with chemical structures similar to their halogenated counterparts may have a positive effect in their biodegradation, through the induction of metabolic enzymes capable of acting on their metabolism. For example, a *Burkholderia sp.* strain was capable of metabolising a group of mono- and di-chlorophenols in the presence of phenol as a growth-supporting substrate, though for highly substituted chlorophenols, such as trichlorophenols and pentachlorophenol this strategy was inefficient (De Los Cobos-Vasconcelos et al., 2006). This may be due to the alteration of molecular steric and biochemical properties that are associated with increasing halogenation of organic compounds, which may cause differences in enzyme recognition and, consequently, substrate interaction. The results obtained in our study indicate that the enzymatic mechanisms involved in the degradation of acetate are not efficient

in the biodegradation of DFA or TFA. On the other hand, the addition of acetate to the cultures fed with DFA or TFA could also have benefited the biodegradation of these compounds by stimulating microbial growth, as reported for other organofluorines (Amorim et al., 2014; Carvalho et al., 2016) which was not verified in this study.

In order to understand how biodegradation is affected when two FAs are simultaneously fed, MFA was supplemented to microbial inocula in mixture with DFA or TFA. MFA defluorination was found to be negatively affected by the presence of DFA in the culture medium, while TFA did not seem to exert any effect in the biodegradation of this compound. This negative influence in MFA defluorination may be associated with an enzymatic inhibition, as MFA and DFA share greater stereochemical similarities than MFA and TFA. This could allow DFA to bind to the active site of the enzyme that metabolises MFA, preventing the binding of this compound to the enzymatic system, thus blocking its action and inhibiting defluorination. As the trifluoromethyl moiety of the TFA molecule has a higher steric bulk than DFA, the MFA degrading enzyme may have a higher capacity to discriminate between these two compounds, and so inhibition does not occur. To the best of our knowledge, the inhibitory effect of DFA in the metabolism of MFA had never been reported before. The results obtained with MFA fed in mixture with DFA or TFA also suggest that the metabolic enzymes responsible for the biodegradation of MFA are selective for this compound and, thus, not able to attack DFA or TFA. This selective MFA catabolism has been reported before by Donnelly and Murphy (2008). The authors isolated a fluoroacetate dehalogenase from *Pseudomonas fluorescens* strain and found that the enzyme was highly selective for MFA and not capable of metabolising DFA and TFA. This is a clear example of the impact that the degree of fluorination may have in the microbial metabolism of fluorinated compounds.

A total of 13 bacterial strains with the capacity to degrade MFA as sole carbon source were isolated from the different MFA-degrading cultures. Taxonomic identification of these strains revealed several microbial species not linked before with the biodegradation of MFA. Some of these species belong to the *Pseudomonas* genus, which, according to previous studies, is known to accommodate a number of MFA-degrading strains (Goldman, 1965; Donnelly and Murphy, 2008). The bacterial isolates identified as *Comamonas testosteroni*, *Variovorax paradoxus* and *Delftia acidovorans*, all belonging to the *Comamonadaceae* family, were also

capable of degrading MFA as sole carbon source, being isolated from all MFA-degrading cultures, independently of their environmental origin. *D. acidovorans* (formerly *Moraxella sp.*) is the only microbial isolate obtained in this study that has been demonstrated before to degrade MFA (Kawasaki et al., 1992; Sota et al., 2002; Kurihara and Esaki, 2008). *C. testosteroni* and *V. paradoxus* have never been associated with the biodegradation of MFA, but their capacity to degrade other recalcitrant compounds, including several chlorinated aromatics, has been described before (Sylvestre, 1995; Boon et al., 2000; Bathe et al., 2009; Satola et al., 2013). On the other hand, *H. frisingense* has never been implicated in the biodegradation of environmental contaminants, to the best of our knowledge.

According to the literature, defluorination of MFA is catalysed by fluoroacetate dehalogenase (Goldman, 1965; Kawasaki et al., 1992; Kurihara et al., 2000). As the genetic expression of this enzyme generally occurs at the plasmidic level, it is possible that horizontal transfer of this genotype may have occurred in the MFA-degrading bacterial communities, which may have contributed to the significant number of bacterial strains capable of degrading this compound obtained in our study (Kawasaki et al., 1981; Kawasaki et al., 1992; Sota et al., 2002; Kurihara and Esaki, 2008). The combination of all MFA-degrading isolates proved to be ineffective in the metabolism of DFA and TFA, namely concerning defluorination of these compounds, reinforcing the conclusion that the enzyme responsible for the defluorination of MFA is unable to act on its di or tri-fluorinated counterparts.

Overall, the results obtained in this study call the attention to the recalcitrant nature of DFA and TFA, as well as to the potential deleterious effects that their continuous release into the environment may have. Though literature studies show that TFA causes no or slight toxic effects in the environment, its increasing environmental release is expected to cause accumulation of this compound, especially in aqueous resources, which may lead to unknown consequences. The effects of the environmental accumulation of DFA are not yet known, but its resistance to biodegradation together with the fact that it may interfere in the degradation mechanisms of defluorinating enzymes, deserves further attention. The inhibition of MFA defluorination caused by the addition of DFA, verified in our experiments, must be taken into consideration regarding the biological removal of mixtures of structurally related fluorinated compounds.

5. Conclusion

The work developed in this study showed that MFA can be metabolised by several bacterial strains from different environmental sources, and that the mechanisms responsible for its catabolism of do not apply in the biodegradation of its di and tri-fluorinated counterparts. Most of the obtained MFA-degrading isolates have not been linked before to the biodegradation of MFA, expanding the range of known microbial species capable of metabolising this fluoroaliphatic compound. Under aerobic conditions, DFA and TFA were recalcitrant to microbial degradation and co-supplementation with the structurally related and more easily degradable substrates, acetate and MFA, had no effect in their biodegradation. These results indicate that the degree of fluorination of fluoroaliphatic compounds significantly influences their biological degradation. When present in mixture, DFA inhibited MFA defluorination, while TFA did not produce any negative effect, a result that, to our knowledge, had never been reported. Such interactions should be taken into account when considering the biodegradation of mixtures of structurally similar fluorinated compounds. Overall, this work emphasizes the recalcitrant nature of DFA and TFA and the potential negative interactions induced by mixtures of fluoroorganics. The persistence and accumulation of FAs in the environment is a relevant issue and may potentially lead to ecosystems disturbances.

CHAPTER 3

BIODEGRADATION OF THE VETERINARY ANTIBIOTICS ENROFLOXACIN AND CEFTIOFUR AND ASSOCIATED MICROBIAL COMMUNITY DYNAMICS

(submitted to SCIENCE OF THE TOTAL ENVIROMENT)

1. Introduction

Veterinary drugs are commonly used to treat numerous animal diseases. Antibiotics constitute one of the most representative groups of these pharmaceuticals, being used not only for the treatment and prevention of diseases, but also for the promotion of animal growth and improvement of the nutritional value of animal-based foodstuffs, despite the legal restrictions concerning these latter applications (Cromwell, 2002; Li et al., 2011).

The overuse of veterinary drugs has contributed to the emergence of these products in several environmental compartments, essentially as a result of the employment of contaminated livestock waste as natural fertilizers (Loke et al., 2000; Tasho and Cho, 2016). In addition, these drugs are also released in the environment through wastewater treatment plants (WWTPs) effluents, because WWTPs are, in most cases, not capable of dealing with this type of contaminants, resulting in incomplete or even no removal of these compounds from agro-industrial effluents (Corcoran et al., 2010).

Pharmaceuticals may be released to the environment in their parental form or as metabolites, including some biologically active ones, and, since they are designed to induce specific physiological and biochemical effects on their target organisms, the environmental presence of these compounds can cause a wide range of toxic effects (Sarmah et al., 2006). For the particular case of antibiotics, their environmental presence may also promote the selection of antibiotic-resistant microorganisms (Martinez, 2009). Fluoroquinolones (FQ) and cephalosporins (CP) are two of the most widely used antibacterial pharmaceuticals worldwide. In 2012, the consumption in Europe of both FQ and CP accounted for over 20% of the total antibiotics consumption (Weist et al., 2014). FQ are piperazinyl derivatives of the N-heterocyclic antibacterial compounds designated as quinolones (Felczak et al., 2014). Their mode of action relies on the ability to inhibit the activity of topoisomerases type II and IV, key enzymes in DNA replication, which leads to the blockage of microbial cell multiplication (Hu et al., 2007). CP are semi-synthetic analogous of the naturally-produced cephalosporin-C (Rex and Susan, 2002). Being a class of β-lactam antibiotics, their antibacterial activity resides in their capability to disrupt peptidoglycan biosynthesis affecting bacterial-cell integrity (Rex and Susan, 2002). Both classes of antibiotics have a broad-spectrum activity towards several aerobic and anaerobic pathogens. FQ have been widely reported to occur in both terrestrial and aquatic ecosystems in trace concentrations, typically ranging

from ng L^{-1} to µg L^{-1}, though concentrations of several mg L^{-1} have also been reported (Picó and Andreu, 2006; Larsson et al., 2007; Zhang and Li, 2011). Physicochemical properties of CP promote a faster environmental dissipation of these antibiotics, leading to lower residence times of these pharmaceuticals in the environment (Junker et al., 2006) and lower detections. As a consequence of the environmental release of these two classes of antibiotics, an increasing number of microorganisms resistant to these drugs has been reported in the literature (Miranda and Castillo, 1998; Walsh, 2000; Ho et al., 2001; Hooper, 2002; Su et al., 2008), highlighting the importance of studying their biodegradation potential.

In this context, the main objective of this work was to investigate the biodegradation of two veterinary antibiotics representative of the FQ and CP groups, enrofloxacin (ENR) and ceftiofur (CEF), respectively. ENR has been reported to occur in wastewaters, agricultural soils and animal manure, while several metabolites of CEF resultant from animal detoxification have been detected in manure and soils (Rex and Susan, 2002; Zhao et al., 2010; Sim et al., 2011; Li et al., 2014). Degradation of these compounds mainly focuses in physicochemical processes (Sturini et al., 2012; He et al., 2014; Zamanpour and Mehrabani-Zeinabad, 2014; Yang et al., 2016), while less studies are found in the literature concerning their biodegradation (Martens et al., 1996; Wetzstein et al., 1997; Rafii et al., 2009; Erickson et al., 2014). In the present work, biodegradation of ENR and CEF, supplemented individually and in mixture, was investigated using microbial communities from the rhizosphere of plants derived from experimental constructed wetlands used for the treatment of livestock wastewaters contaminated with these antibiotics (100 µg L^{-1}) (unpublished data). The effect of the target antibiotics in the microbial dynamics of the degrading cultures was also studied through metagenomics analysis.

2. Materials and methods

2.1. Enrichment of microbial degrading cultures

Microbial cultures capable of degrading ENR and CEF were obtained by selective enrichment of inoculated culture medium with the target antibiotics, supplemented either individually or in mixture, and using acetate as a co-substrate. Rhizosphere sediment samples obtained from experimental constructed wetlands previously

designed for the treatment of livestock wastewaters contaminated with the target antibiotics were used as inocula. Enrichments were conducted in duplicate, in batch mode and under aerobic conditions, during ca. 5 months. For that, 250 mL flasks containing 50 mL of sterile minimal salts medium (MM) were inoculated with 5 g of sediment and fed with the target antibiotics at the concentration of 1 mg L^{-1} and acetate at the concentration of 400 mg L^{-1}. MM contained (per liter):): $Na_2HPO_4 \cdot 2H_2O$ 2.7 g, KH_2PO_4 1.4 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g and 10 mL of a trace elements solution with the following composition, per litre: $Na_2EDTA \cdot 2H_2O$ 12.0 g, NaOH 2.0 g, $MnSO_4 \cdot 4H_2O$ 0.4 g, $ZnSO_4 \cdot 7H_2O$ 0.4 g, H_2SO_4 0.5 mL, Na_2SO_4 10.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 2.0 g, $CuSO_4 \cdot 5H_2O$ 0.1 g and $CaCl_2$ 1.0 g. Microbial cultures were incubated in a rotary shaker (130 rpm), at 25ºC and protected from light. Acetate was fed to the cultures twice a week. Every 3 weeks, 25 mL of the microbial cultures were transferred to new flasks containing equal volume of MM and re-fed with the target antibiotics and acetate. Every week, cultures were transferred to new flasks to assure appropriate aerobic conditions. Microbial enrichment was followed by monitoring microbial growth, fluoride ion release for ENR and by measuring the concentration of ENR and CEF in the culture medium.

2.2. Biodegradation of different concentrations of ENR and CEF

After the enrichment period, biodegradation of the target antibiotics was investigated for concentrations of 2 and 3 mg L^{-1}. For that, 250 mL flasks containing 25 mL of MM and 25 mL of the microbial cultures enriched in the previous phase (section 2.1) were initially supplemented, in triplicate, with the target antibiotics, each at a concentration of 3 mg L^{-1} (supplemented individually and in mixture) and acetate (supplemented twice a week at a concentration of 400 mg L^{-1}). Cultures were incubated for a 3 weeks period in the same conditions used during the enrichments (section 2.1). Aerobic conditions were maintained in the microbial cultures as described previously. Biodegradation was monitored by analysing microbial growth, fluoride ion release for ENR and antibiotics concentrations in the culture medium. At the end of the 3 weeks period, microbial cultures were again diluted to half of their volumes and doped a second time with the target antibiotics at the same concentration (3 mg L^{-1}) and acetate (supplemented in the same regime). Biodegradation was followed for an additional 3 weeks period, after which

the same procedure was repeated to test the biodegradation of the antibiotics at a lower concentration, each at 2 mg L^{-1}.

In parallel with the biodegradation experiments, two sets of abiotic controls were established. One consisted in sterile MM supplemented with ENR and CEF, both individually and in mixture, at a concentration of 2 mg L^{-1}, and the other consisted in sterile MM inoculated with autoclaved microbial consortia obtained from the enrichment phase (initial optical density at 600 nm of 0.1), supplemented with 2 mg L^{-1} of the target antibiotics. Controls were established in triplicates and incubated for one month in the same conditions of the degradation experiments.

2.3. Analytical methods

Biomass growth was monitored by reading the absorbance of culture samples at 600 nm, in a spectrophotometer (V-1200, VWR International, USA).

Fluoride ion release was measured as an indicator of ENR defluorination. The concentration of fluoride ion in solution was analyzed, after centrifuging samples at 13000 rpm for 15 min, with a fluoride-selective electrode (Crison 9655 C, Crison Instruments, S.A., Spain). Prior to sample analysis a calibration curve was obtained using standard solutions of sodium fluoride (0.001 to 1 mM) prepared in MM. A total ionic strength adjustment buffer (TISAB III) was supplemented to the samples and standards in a 1:10 ratio.

CEF and ENR were analyzed in the supernatant of the culture samples by HPLC. Supernatants were obtained through centrifugation at 13000 rpm for 15 min. Separation of the target antibiotics was performed in a C18 Luna column (150 x 4.6 mm) from Phenomenex, coupled to a Beckman Coulter HPLC equipped with a diode array detector (module 128) and an automatic sampler (module 508). Chromatographic conditions were the same as described elsewhere (Cavenati et al., 2012). ENR was screened at 280 nm, while CEF was detected at 290 nm. The analytical detection limit (LOD) for all the target antibiotics was 0.1 mg L^{-1}. Standard solutions of the antibiotics were prepared in MM (0.1 - 6 mg L^{-1}) and used to obtain calibration curves prior to every analysis.

2.4. Analysis of the structure of the microbial communities

The effect of the enrichment process with the target antibiotics in the different degrading cultures was investigated by comparing the structure of the microbial communities of the soil samples used as initial inocula with that of the microbial cultures obtained at the end of the biodegradation experiments. DNA from the soil samples used as inocula for the experiments was extracted from 0.5 g (wet weight) of homogenized sediment using PowerSoil® DNA Isolation Kit from MOBIO Laboratories, Inc., according to the manufacturer's instructions. DNA from the degrading cultures was obtained using a standard phenol-chloroform extraction method, as described elsewhere (Sambrook et al., 1989). Briefly, microbial biomass was harvested by centrifuging 1 mL culture aliquots and removing the supernatant, to which it was added STE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 8.0), sodium dodecyl sulphate (20%) and proteinase K (20 mg mL^{-1}). The mixture was incubated overnight at 56ºC with gently shaking. After the incubation period, samples were transferred to Light Phase Lock Gel tubes (5 Prime Inc., Hamburg, Germany) to which phenol: chloroform: isoamyl (25:24:1) and chloroform: isoamyl (24:1) alcohols were sequentially added, with intercalated centrifugations (14 000 rpm for 3 min) to separate the aqueous and organic phases. Finally, the obtained DNA was concentrated through ethanol precipitation and the resulting pellets were air dried in sterile conditions, at room temperature. DNA extracts from soil samples and from the degrading-microbial consortia were then dissolved in 50 µL of sterilized water.

Structure of microbial communities in different samples was assessed by Illumina Miseq sequencing of the 16S rRNA gene. Fusion primers consists of adaptor A or B, key sequence, barcode and template specific sequences were used in this study. Specifically, the V4-V5 region of the bacterial 16s rRNA gene was amplified by Polymerase Chain Reaction (PCR) with the forward primer 515F (5'-GTGCCAGCMGCCGCGG-3') and the reverse primer 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), and a 12 bp adaptor sequence was attached to the 5' end of 515F. The 50 µL PCR reaction mixture contained 1 x PCR buffer (Mg2+ plus), 0.2 mM of each deoxynucleoside triphosphate, 0.4 mM of each primer, 1.25 U of TaKaRa Taq HS polymerase (TaKaRa Biotech, Dalian, China) and 1 µL template DNA. The PCR amplification program included initial denaturation at 94˚C for 5 min, followed by 32 cycles at 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 45 s, and a final extension at 72˚C for 5 min. Amplified products were subjected to

electrophoresis using a 1.8% agarose gel. Amplicon bands with a suitable size (475 bp) were excised from the gel and purified with an agarose gel DNA purification kit (TaKaRa Biotech, Dalian, China). All of the purified amplicons were then combined in equimolar amounts and submitted to high-throughput sequencing on an Illumina MiSeq pyrosequencer. The MiSeq sequencing data was analysed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://qiime.org/). Briefly, low quality sequences, which have lengths of <200 bp, an average quality score of <25 and primer mismatches were trimmed and the barcodes were determined to assign sequence reads to the proper samples. Then, the chimeras were detected using the UPARSE algorithm based on a database of chimera-free sequences. The sequences, which were assigned to a mitochondrial or chloroplast origin were eliminated with the Metaxa software tool and the V4–V5 region was extracted with the V-Xtractor software tool.

2.5. Statistical analysis

For the biodegradation experiments, replicates of samples were analyzed independently and mean values and corresponding standard deviations were calculated. For the metagenomics analysis, composite samples were used on all experimental conditions.

Statistical analysis was performed using the software STATISTICA version 12 (StatSoft, Inc., 2013). For antibiotic removals and ENR defluorination, statistically significant differences were evaluated through a parametric Student's t-test, using mean values and corresponding standard deviations of the replicates. Statistical significance was assumed when the p-value was below or equal to 0.05.

Metagenomics profiles were analyzed using PRIMER 6 software package (v. 6.1.11) (Clarke and Gorley, 2006). Bacterial richness and diversity index (Shannon Index) were calculated based on the different number of OTUs and relative abundances of the different OTUs. Normalization of the metagenomics profiles was performed using the presence/absence pre-treatment function and, afterwards, a resemblance matrix was created using the Bray-Curtis similarity method, from which a hierarchical cluster was constructed using group average method. SIMPROF test was used to detect differences among generated clusters.

3. Results

3.1. Biodegradation of ENR and CEF

To investigate the biodegradation of ENR and CEF, an enrichment period of 5 months was conducted using sediment samples obtained from experimental constructed wetlands treating livestock wastewaters contaminated with these antibiotics. Acetate was added to the cultures as a growth supporting substrate. The purpose of this acclimation phase was to allow the adaption of the microbial communities to each antibiotic.

During the first nine weeks of the enrichment phase, both microbial growth and defluorination were not followed in the cultures due to the interference of the sediment inocula in the analysis of these parameters. According to Table 3, nine weeks after the beginning of the enrichments, biodegradation of ENR (based on fluoride release) in the cultures fed individually with this compound and in mixture with CEF was ca. 53 % and 65 %, respectively. In these microbial cultures, ENR was gradually defluorinated along each feeding period of 3 weeks, with most of fluoride being released in the last two weeks (Table 3). These results remained very similar until the end of the enrichment phase (data not shown), and the complete ENR defluorination was never achieved. During this phase, CEF was found to be always completely removed from the culture media, while ENR removals ranged between 45 and 55 % when supplemented individually or in a mixture, respectively. Along the enrichment phase, microbial cultures always had an increase on their microbial densities (supported by the addition of acetate), showing a gradual increment over time in their optical density (OD) (data not shown).

After the enrichment period, microbial cultures were tested for their capacity to degrade ENR and CEF at the concentrations of 2 and 3 mg L^{-1}. Microbial cultures were initially fed with the highest concentration, 3 mg L^{-1}, to test their robustness to degrade the target antibiotics. In these conditions, defluorination of ENR sharply decreased, being obtained values of ca. 4 and 3 % in the cultures fed with ENR and with a mixture of ENR and CEF, respectively (Fig. 6). However, based on antibiotics analysis in supernatant culture medium, these microbial cultures were able to consume ca. 40 % of the supplemented ENR (Fig. 6).

Table 3. Defluorination performance along a feeding period of 21 days, obtained nine weeks after the beginning of the enrichment phase, for ENR supplied individually and in mixture with CEF, at the concentration of 1 mg L^{-1}

Time (days)	% of ENR defluorination	
	ENR[a]	ENR + CEF[a]
7	18 ± 1	6 ± 3
14	24 ± 6	46 ± 5
21	53 ± 2	65 ± 3

Note: [a]Results are expressed as the mean of duplicates ± standard deviation

Removal efficiencies of 100% were always observed for CEF, both in the cultures supplemented individually with this antibiotic and in the cultures fed concomitantly with ENR (data not shown).

When the cultures were fed with 2 mg L^{-1} of the target antibiotics, ENR biodegradation performance improved, namely its defluorination, despite the attained values being far below those obtained during the enrichment phase with 1 mg L^{-1}. Under these circumstances, similar ($p>0.05$) defluorination efficiencies of ENR were achieved in the cultures fed with ENR and with a mixture of the two antibiotics, with values of 22 and 16 % of defluorination being obtained, respectively. At this concentration, ENR removals were fairly constant, showing no significant differences ($p>0.05$) to the ones obtained when this antibiotic was fed at 3 mg L^{-1} (Fig. 6). Removal efficiencies of 100 % were again observed for CEF, showing no significant differences in function of its concentration or the concomitant presence of ENR.

The increase in antibiotics concentrations did not affect microbial growth, being achieved OD increments similar to the ones observed in the enrichment phase (data not shown). Analysis of the antibiotics in the supernatant of the microbial cultures supplemented with 2 or 3 mg L^{-1} of ENR (both individually and in mixture with CEF) revealed the presence of two metabolites, though in concentrations below the LOQ, identified as ciprofloxacin (CIP) and norfloxacin (NOR) by comparison with the corresponding standard solutions.

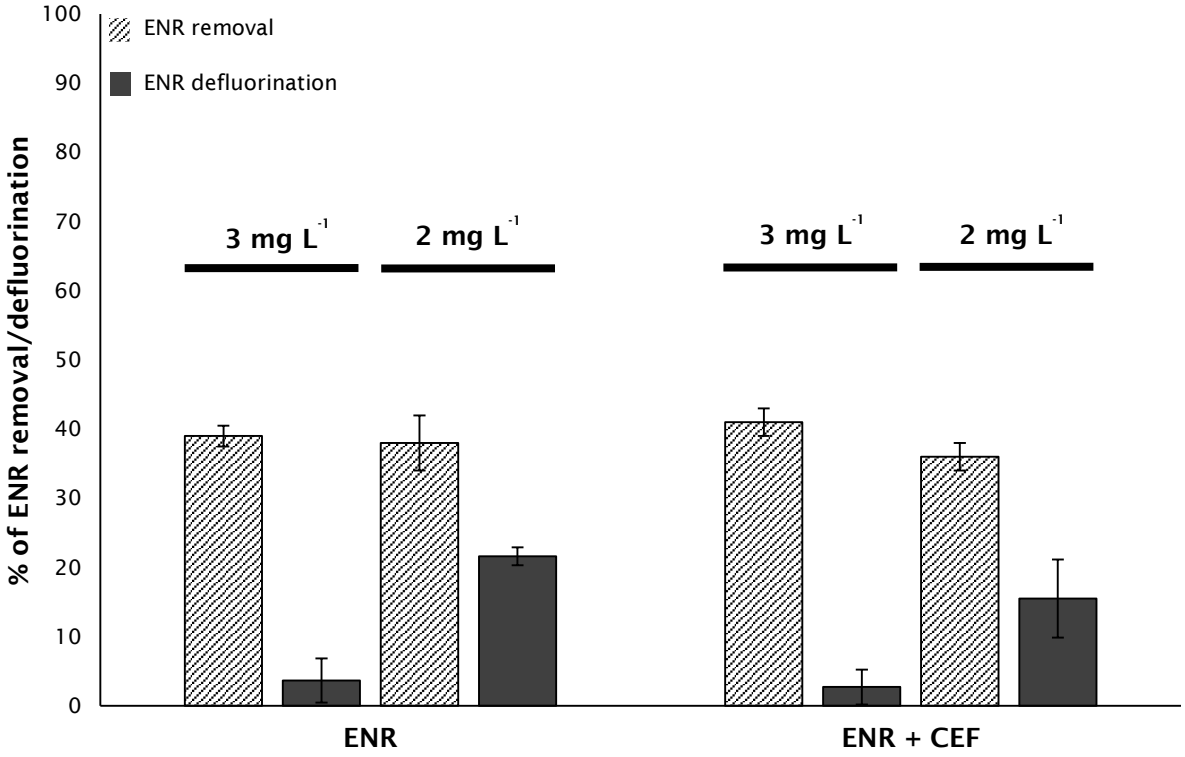


Figure 6. Biodegradation of ENR, supplied individually and in a mixture with CEF for the concentrations of 3 and 2 mg L^{-1}. Results are expressed as the mean of triplicates and error bars are relative to standard deviation.

Comparing total removal with abiotic controls (Fig. 7), it was observed that a substantial amount of CEF was removed abiotically, having also a considerable capacity to adsorb to microbial cells. After 30 days of incubation, ca. 39 % and 37 % of CEF was removed in the controls with no cells and in the controls containing autoclaved consortia, respectively (Fig. 7). In contrast, ENR showed no removal or defluorination in the controls without cells, having only a slight potential for cell adsorption as evidenced by the ca. 6 % removal obtained in the controls with autoclaved cultures (Fig. 7). The adsorption behaviour of both antibiotics did not seem to be influenced by their simultaneous presence, as no significant differences were observed in this condition ($p>0.05$) (Fig. 7).

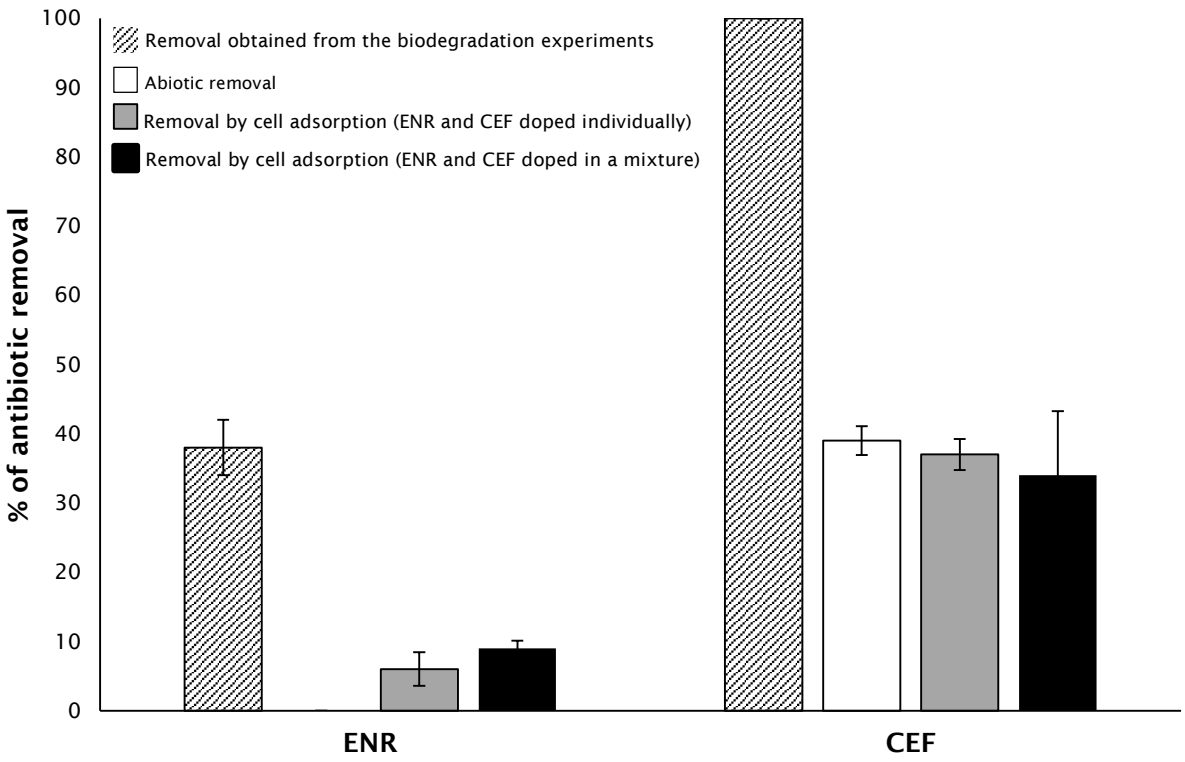


Figure 7. Removals of ENR and CEF obtained in different experimental conditions, for the concentration of 2 mg L^{-1}. Results are expressed as the mean of triplicates and error bars show standard deviation.

3.2. Analysis of microbial communities' dynamics

To investigate the effect of the enrichment process with the target antibiotics in the microbial communities used as inocula for the degrading experiments, microbial compositions at the beginning and at the end of the experiments were compared by metagenomics analysis.

Cluster analysis based on the Bray-Curtis similarity method showed that the enriched communities are significantly different from the initial ones and that the mode of antibiotics supplementation (individually or in mixture) did not influence the structure of the enriched microbial community (Fig. 8). This trend is also supported by the clear differences determined among the initial and the enriched consortia, with the latter showing lower microbial diversity and abundance (Table 4).

Concerning microbial structure, five dominant phyla were found in the initial communities: *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes* and *Chloroflexi*, accounting for over 80% of the structure of the communities of the initial inocula (Fig. 8). Microbial enrichments with the target antibiotics caused a

clear decrease in the abundance of microorganisms belonging to the phyla *Firmicutes* and *Actinobacteria*, while the phyla *Proteobacteria* and *Bacteroidetes* gained expression, representing between 80 to 90% of the entire microbial communities of the final consortia (Fig. 8). Cultures enriched with ENR and with a mixture of ENR and CEF also showed an increase in microorganisms belonging to the phylum *Spirochaetae* (Fig. 8).

Table 5 shows the relative abundance of the most represented taxonomic groups identified in the initial microbial communities and in the antibiotics enriched microbial cultures. Enrichments with ENR and CEF supplied individually and in a mixture, led to the selection of microorganisms belonging to the taxonomic groups *Rhizobiales, Betaproteobacteria* and *Comamonadaceae* and to the loss of *Acidomicrobiales, Anaerolineaceae* and *Xanthomonadaceae* (Table 5). The bacterial genus *Dysgonomonas* showed an increased expression with the antibiotics enrichment, while the genus *Clostridium* lost representation in all the final microbial communities (Table 5). The *Betaproteobacteria* class was the most representative group in the enriched microbial communities, with the highest number of unidentified species (ranging from 33.7 to 36.5 %).

Despite the general shifts observed at the genus level, for all the enriched microbial communities, metagenomics analysis showed that the mode of antibiotics supplementation led to the selection of specific genera. For the cultures enriched with ENR, the selection of the genera *Flavobacterium* (20.8 %) and *Achromobacter* (8.4 %) was observed, while the genera *Stenotrophomonas* (12.8 %) and *Chryseobacterium* (29.3 %) increased their expression in the microbial cultures enriched with CEF and with a mixture of ENR and CEF, respectively (Table 5).

4. Discussion

There are several physicochemical processes capable of removing FQ and CP from environmental matrices, but only a few biotic mechanisms have been described for their degradation (Sturini et al., 2012; He et al., 2014; Karlesa et al., 2014; Yang et al., 2016). The potential of environmental microorganisms to biodegrade these antibiotics is yet to be properly elucidated and the work developed in this study intends to shed some more light in this respect.

Table 4. Diversity and abundance indexes of the initial inocula and microbial communities enriched with the target antibiotics

Microbial community	Richness[a]	Diversity[b]
$ENR_{initial}$	368	3.967
$CEF_{initial}$	377	4.401
$ENR + CEF_{initial}$	410	4.757
ENR_{final}	143	2.290
CEF_{final}	145	2.253
$ENR + CEF_{final}$	121	1.994

Note: [a]number of OTU; [b]Shannon diversity index (H').

Microbial acclimation constitutes an important process in the biodegradation of environmental pollutants, including pharmaceutical compounds. Liao et al. (2016), compared the biodegradation performances of CIP by non-acclimated and acclimated microbial communities, and showed that this antibiotic was more readily removed by acclimated consortia. The 5-months enrichment phase conducted in this study certainly had an important role in the biodegradation performance of the target antibiotics at the tested concentrations, allowing the selection of microorganisms with higher potential to deal with these compounds. This is supported by the observed shifts in the diversity and richness of the microbial communities after a prolonged time of enrichment.

ENR was shown in this study to be metabolized by the enriched microbial consortia, though complete defluorination and removal of this antibiotic has never been achieved. Microbial defluorination of this antibiotic was significantly influenced by its concentration, with defluorination being higher when the antibiotic was supplemented at 1 mg L^{-1} and declining markedly with the increase of ENR concentration. However, under these circumstances, the removal efficiency of ENR did not change significantly, suggesting that fluoride release constitutes a limiting step in the biodegradation of ENR.

In the cultures fed with ENR (both individually and in mixture with CEF), the metabolites CIP and NOR were consistently detected, but it remained unclear if their production was a consecutive event or if it corresponded to independent metabolic pathways.

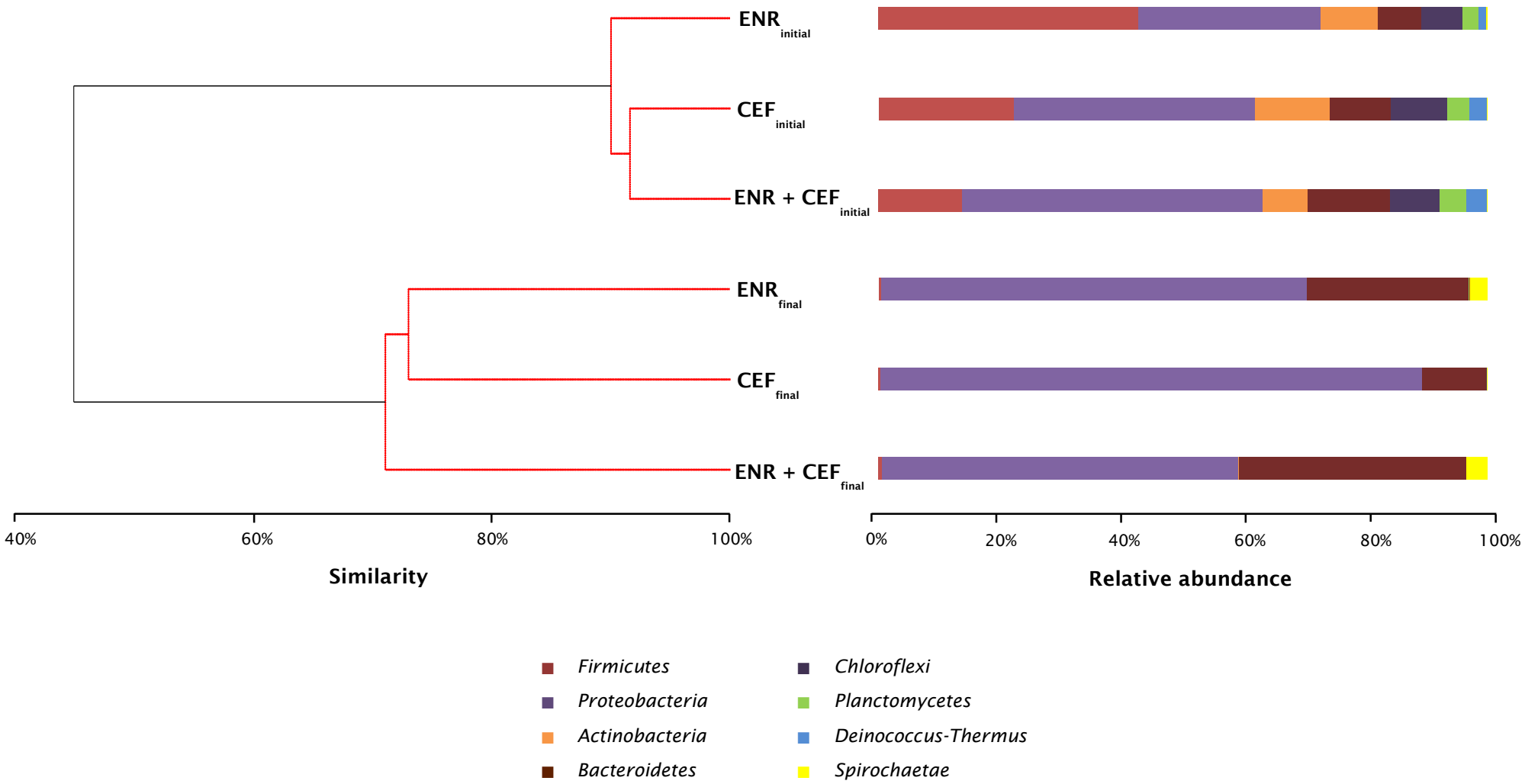
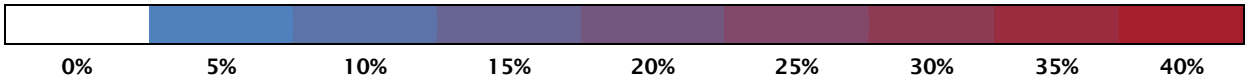


Figure 8. Cluster analysis based on Bray-Curtis similarity of metagenomics profiles of microbial communities and relative abundance of the different bacterial phyla at the beginning and at the end of the biodegradation experiments. Dashed lines indicate samples that are similar ($p>0.05$) according to the SIMPROF test.

Table 5. Metagenomics profiles of the initial inocula and enriched consortia, showing the relative abundance of each taxonomic group in the communities (relative abundances below 2 % were not considered)

Phylum	Class	Order	Family	Genus	$ENR_{initial}$	$CEF_{initial}$	$ENR+CEF_{initial}$	ENR_{final}	CEF_{final}	$ENR+CEF_{final}$
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*			15%	20%	10%			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Dysgonomonas*				5%	15%	20%
	Flavobacteriia	*Flavobacteriales*	*Flavobacteriaceae*					5%		10%
				Flavobacterium				20%		
				Chryseobacterium						30%
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*		10%	15%	15%			
Deinococcus-Thermus	*Deinococci*	*Deinococcales*	*Trueperaceae*	*Truepera*		10%	10%			
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*		5%					
			Clostridiaceae	*Clostridium*	20%	20%	15%			
			Peptostreptococcaceae		15%	5%				
	Erysipelotrichia	*Erysipelotrichales*	*Erysipelotrichaceae*	*Turicibacter*	10%					
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*						5%	15%	15%
			Bradyrhizobiaceae	*Bosea*				5%		
			Brucellaceae						15%	
			Phyllobacteriaceae	*Mesorhizobium*	10%					
			Xanthobacteraceae					15%		
	Betaproteobacteria				5%	15%	15%	20%	20%	20%
		Burkholderiales	*Alcaligenaceae*	*Achromobacter*				15%		
			Comamonadaceae					15%	15%	5%
				Variovorax					15%	
		Neisseriales	*Neisseriaceae*				5%			
	Gammaproteobacteria	*Xanthomonadales*	*Xanthomonadaceae*		10%	15%	15%			
				Arenimonas		5%	5%			
				Stenotrophomonas					15%	
Spirochaetae	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*				5%		5%



Nonetheless, the identification of these metabolites in the cultures supplemented with ENR suggests that, at least, part of the molecule is not immediately subjected to an initial defluorination step, as the identified metabolites (NOR and CIP) also bear a fluorine atom in their structures. The metabolite CIP has been reported before to be involved in the biodegradation of ENR by fungal species, being produced by deethylation of the ENR piperazine ring (Wetzstein et al., 2006). On the other hand, to the best of our knowledge, NOR has never been reported before as an intermediary metabolite of ENR biodegradation. Further biodegradation of these two fluorinated metabolites are described to proceed via attack to the piperazine ring, with fluoride removal occurring afterwards through a hydroxylation reaction (Amorim et al., 2013; Liao et al., 2016). In this study, it is possible that biodegradation of ENR follows a similar pathway, in which the following steps may occur: (i) initial conversion of ENR into CIP and/or NOR; (ii) loss of the piperazine moiety in both CIP and NOR, resulting in other metabolites still bearing fluorine in their structure; (iii) defluorination of these fluorinated products by hydroxylation. This chain of reactions is expected to generate smaller and simpler molecules, with less antibacterial activity that may be more easily used as carbon sources by environmental microorganisms (Wetzstein et al., 2009; Liao et al., 2016). While defluorination of ENR may not be an immediate catabolic step, it may contribute to the inactivation of its bactericidal properties, as it has been shown before for other FQ (Carvalho et al., 2016).

Studies on the biodegradation of ENR indicate that this antibiotic is mainly degraded by fungi. *Gloeophyllum striatum* was reported to metabolize 5 and 10 mg L^{-1} of ENR, but complete degradation has never been achieved in a period of eight weeks, while *Mucor ramannianus* was able to degrade ca. 79 mg L^{-1} of ENR in a 21 days period, though no information on defluorination of the molecule is given in the study (Martens et al., 1996; Wetzstein et al., 1997; Parshikov et al., 2000; Wetzstein et al., 2006). A wide network of metabolites resulting from the different biodegradation pathways of ENR by *G. striatum* has been identified, with a small portion of these metabolites being non-fluorinated congeners of the parental compound that were generated as a primary metabolic step through a hydroxylation reaction (Wetzstein et al., 1997; Karl et al., 2006). Parshikov et al. (2000) identified three fluorinated ENR metabolites produced by *M. ramannianus*, with one of them being implicated before in the biodegradation

of ENR by *G. striatum* and another being involved in the biodegradation of CIP also by *M. ramannianus* (Wetzstein et al., 1997; Parshikov et al., 1999). Recent studies on FQ biodegradation are mainly focused in second-generation FQ, such as NOR, CIP and ofloxacin, though bacterial degradation of moxifloxacin, a fourth generation FQ, has also been investigated (Girardi et al., 2011; Amorim et al., 2013; Maia et al., 2014; Carvalho et al., 2016; Liao et al., 2016). These studies indicate that the capacity to degrade FQ depends largely on the microorganisms involved and the associated growth conditions. For example, CIP has shown to be recalcitrant, along 93 days, in both aquatic and soil ecosystems at a concentration of 20 mg L^{-1}, with only minimal degradation (0.9 %) being found in soil after that period of time (Girardi et al., 2011). However, higher biodegradation performances for this same antibiotic have been verified, when present in lower concentration ranges. Microbial communities isolated from a biological activated carbon filter system designed for the treatment of lake water contaminated with antibiotics were capable of growing in the presence of 10 mg L^{-1} of CIP as a sole substrate (Liao et al., 2016). Also, an *Alphaproteobacteria* strain, *Labrys portucalensis* strain F11, was able to convert 85% of 1 mg L^{-1} of CIP in 28 days (Amorim et al., 2013). This bacterial strain was also capable of metabolizing other FQ, including ofloxacin, NOR and moxifloxacin, in concentrations ranging from 1 to 10 mg L^{-1} (Amorim et al., 2013; Maia et al., 2014; Carvalho et al., 2016). In these latter studies, and similarly to our results, defluorination was also shown to be a limiting step in the microbial degradation of the tested FQ.

Microbial cultures supplemented with CEF were always capable of completely removing this compound from the culture medium, independently of its concentration or the concomitant presence of ENR. Although a part of this removal was due to abiotic processes, these results are in agreement with other literature studies on the biodegradation of CEF. A wide group of anaerobic bacterial strains obtained from bovine waste was shown to be able to fully remove 5 mg L^{-1} of this antibiotic within 24 to 120 hours (Rafii et al., 2009). Biodegradation of 10 mg L^{-1} of CEF by fecal microorganisms has also been reported (Li et al., 2011; Erickson et al., 2014). Among these microorganisms, a *Bacillus cereus* was capable of growing with concentrations of this antibiotic above 100 mg L^{-1} (Erickson et al., 2014). Some of these microorganisms were found to be capable of expressing β-lactamases, a group of enzymes that play a

fundamental role in the complete degradation of CEF (Rafii et al., 2009; Erickson et al., 2014). It is possible that part of the removal of CEF obtained in this work is a result of similar enzymatic activities, as the expression of β-lactamases in environmental microorganisms is a very common phenotype (Rafii et al., 2009; Bush and Jacoby, 2010; Erickson et al., 2014). It is reported that one of the primary targets in CEF biodegradation is the β-lactam moiety, a mechanism that may also have occurred in CEF degradation by the microbial consortia enriched in this work (Li et al., 2011). This reaction may be responsible for a considerable reduction of CEF antibacterial properties, as the antibiotic potential of CP rely heavily on the integrity of their lactam ring (Rex and Susan, 2002).

In the microbial cultures supplemented simultaneously with ENR and CEF, biodegradation performances of these compounds were very similar to the ones obtained in the cultures fed individually with these antibiotics. This indicates that the metabolic mechanisms responsible for CEF removal do not affect ENR degradation and vice-versa, and that the enzymes responsible for the metabolism of these two drugs are likely to be distinct. This result is highly relevant, as it suggests that the concomitant environmental presence of these two antibiotics will not hinder their microbial removal.

Both biotic and abiotic mechanisms played an important role in the removal of CEF. This is also expected to occur in an environmental scenario, which might explain why CP do not tend to persist in the environment. Two abiotic mechanisms, namely hydrolysis and photolysis, have been reported to be involved in the breakdown of CP, including CEF (Jiang et al., 2010; Li et al., 2011). In this work, abiotic degradation of CEF might have occurred through a hydrolysis mechanism, as the experiments were always conducted in the absence of light. Unlike CEF, abiotic degradation of ENR was found to have a minor role in the removal of this antibiotic, indicating that it was primarily degraded through the catabolic action of the enriched microbial consortia. In addition, abiotic controls with autoclaved consortia also showed that both ENR and CEF tended to bind to microbial membranes, with CEF showing a higher potential. While this may account as a removal mechanism, adsorbed antibiotics may still have been metabolized in the degradation experiments, as adsorption is usually a reversible reaction.

Enrichments with the target antibiotics, supplied individually or in mixture, had a significant effect on the structure and diversity of the microbial communities.

Both individual and simultaneous presence of ENR and CEF is expected to promote microbial selection in the communities, selecting those microorganisms capable of breaking down these compounds. Diversity of all enriched consortia decreased when compared with the corresponding initial communities, which may be a consequence of microbial cultures being exposed to higher antibiotic concentrations and to growth conditions different than those of the experimental systems from where the inoculum samples were derived. However, abundance was markedly higher in the enriched consortia, which may be due to the frequent co-supplementation of microbial cultures with acetate as an easily degradable carbon source, allowing a higher growth of the communities selected by the presence of the target antibiotics. In a study conducted by Fernandes et al. (2015) on the removal of the antibiotics ENR and tetracycline in constructed wetlands microcosms, the authors found that the presence of ENR (100 μg L^{-1}) did not induce significant long-term changes in microbial abundance and diversity, but resulted in significant differences in the microbial community structure. Liao et al. (2016) observed a decrease in microbial abundance but similar diversity indexes (Shannon index) in the presence of CIP. However CIP was supplemented as a sole carbon source, which could explain the lower abundance in the communities, and microbial dynamics was followed along a shorter period of time (28 days), which could have been not enough to trigger significant diversity alterations in the microbial communities. Girardi et al. (2011) has shown that longer exposure periods to CIP (up to 65 days) can cause considerable community shifts.

Overall, two of the most dominant bacterial phyla present in the initial communities, *Firmicutes* and *Actinobacteria*, suffered a considerable decrease in the enriched consortia, with the phyla *Proteobacteria* and *Bacteroidetes*, gaining a higher expression in the enriched communities. In a metagenomics study conducted with CIP, microorganisms belonging to *Proteobacteria* and *Actinobacteria* phyla were mainly selected, whereas *Bacteroidetes* and *Firmicutes* species lost their expression (Liao et al., 2016). The fact that in both studies a selection of microorganisms belonging to the phylum *Proteobacteria*, was promoted, with a special emphasis on *Betaproteobacteria*, suggests that members of this taxonomic group may have an important role in the biodegradation of FQ. Among the phylum *Bacteroidetes*, representation of the genus *Dysgonomonas* increased in the consortia enriched with the target

antibiotics, both individually and in mixture, indicating that this taxonomic group likely has a role in the biodegradation of both ENR and CEF. Liao et al. (2016) also found an increase of *Dysgonomonas* species in CIP-enriched communities, suggesting that microorganisms belonging to this genus may be involved in the biodegradation of FQ. Other bacterial genera selected in the enriched communities were *Flavobacterium, Chryseobacterium, Achromobacter, Variovorax* and *Stenotrophomonas*. These genera have already been associated with the biodegradation of recalcitrant organic compounds, many of them halogenated. For example, *Achromobacter* species have been reported to be involved in the biodegradation of several sulfonamides (Li et al., 2009; Xu et al., 2013; Reis et al., 2014); *Flavobacterium* species have been reported to be capable of degrading the chlorinated pesticide, pentachlorophenol (Hu et al., 1994; Lo et al., 1998); *Variovorax* species were shown to metabolize several derivatives of phthalate and the pesticide linuron (Prasad and Suresh, 2012; Horemans et al., 2013; Prasad and Suresh, 2015) and *Chryseobacterium* and *Stenotrophomonas* species were described to be capable of using a wide range of chlorinated and fluorinated pesticides, such as flubendiamide, tetraclorophenol or DDT (Deng et al., 2015; Jadhav and David, 2016; Pan et al., 2016).

5. Conclusion

In this study, ENR and CEF were degraded at different extents by microbial communities derived from experimental constructed wetlands designed to treat wastewaters contaminated with trace amounts of the two antibiotics. While complete removal of CEF was always achieved, ENR showed to be more recalcitrant. Removal percentages for this latter antibiotic between 40 and 60 % and defluorination percentages between 3 and 79 % were obtained, with biodegradation being affected by the increase in its concentration. The simultaneous supplementation of ENR and CEF did not affect the biodegradation of these antibiotics. Contrarily to what was found for ENR, abiotic mechanisms had a significant role in the removal of CEF, which may be one of the reasons why this antibiotic has a faster dissipation in the environment. Microbial dynamics associated to the enrichments with the target antibiotics revealed a shift in the structure of the microbial communities, with a predominant selection of microorganisms belonging to the phyla *Proteobacteria* (e.g., *Achromobacter,*

Variovorax and *Stenotrophomonas* genera) and *Bacteroidetes* (e.g., *Dysgonomonas, Flavobacterium* and *Chryseobacterium* genera). Overall, this work demonstrated that microorganisms are capable of adapting and responding to the presence of different emergent pollutants, like the antibiotics used in this study, though concentration is a key factor in the biodegradation process. The biodegradation capacity of the tested antibiotics exhibited by the microbial communities enriched in this study suggest that environmental sites contaminated with mixtures of ENR and CEF, where lower concentrations of these contaminants are typically present, are likely to be recovered, at least partially, through bioremediation processes.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

1. General discussion

During the last decades, fluorinated organic compounds have become common environmental contaminants due to their high versatility and favourable properties, being increasingly used in various sectors of our societies. Nowadays, fluoroorganics are amongst some of the most used synthetic compounds in areas such as human and veterinary medicine, agriculture or even in the industrial sector.

While a lot of research and development is being carried out on the industrial production of organofluorine compounds, less efforts are being directed towards the effects of these products on human and environmental health. This is a concerning issue, as the consumption of fluorinated products is not showing a decreasing trend. Also, a lot of these products find themselves in legal grey areas, as their production, application and elimination procedures remain highly unregulated.

Only recently the hazardous nature of organofluorines, including their human toxicity and potential for ecosystem damage, has been acknowledged in the scientific literature. Key et al. (1997) was among the first scientists to address this issue, being also the first author to recognize organofluorines as "ubiquitous environmental contaminants". Since then, other important works have been published, but a big gap of knowledge still exists on the toxicity, environmental impact and biodegradation of fluorinated compounds.

In the last 20 to 30 years, biodegradation studies mainly targeted non-halogenated and chlorinated environmental contaminants, focusing less on organofluorine compounds. Presently, due to the rapid expansion and spread of fluorinated compounds, these are found in the environment as micropollutants, although higher concentrations than those usually reported for emergent contaminants have also been found (Larsson et al., 2007; Piekarz et al., 2007; Harada and Koizumi, 2009).

Knowledge on fluoroorganics biodegradation efficiency, metabolic pathways, as well as on the involved microorganisms is highly relevant, as it is crucial for the design of efficient bioremediation technologies. In this thesis, the biodegradation of different organic fluorinated compounds was investigated, in order to understand their biodegradation potential by environmental microbial communities and to obtain knowledge on the microbial species/microbial communities involved in the biodegradation process. Also, and whenever possible,

insights on the metabolic pathways of the target compounds were given, based on the obtained experimental data.

The first study conducted showed that MFA was readily biodegraded by a wide diversity of environmental bacteria. This capacity has been reported before by other authors and may be due to the existence of an enzyme capable of specifically catalysing the defluorination of this compound. Yet, such specific defluorinating enzymes are not common in the metabolism of organofluorines, and in most cases defluorination occurs as a result of non-specific catabolic reactions. Also, the biodegradation results obtained for MFA, DFA and TFA, showed that the degree of fluorination plays a major role in the recalcitrance of fluorinated compounds. For the case of FAs, this resulted in MFA being completely defluorinated, while DFA and TFA still held the fluorine atoms in their structures. The absence of a proper aerobic biological degradation of DFA and TFA is concerning, as their recalcitrance and environmental dynamics may lead to an increase of these compounds in aquatic ecosystems, where they are likely to persist over time.

The work conducted with ENR revealed that biodegradation of this compound was highly influenced by its concentration, with degradation efficiency decreasing with the increase on the concentration of this compound. Additionally, results showed that defluorination apparently is not a primary step in the biodegradation of this fluoroquinolone, as the metabolic intermediates CIP and NOR, both still bearing fluorine in their structures, were detected in the culture medium. While biological mechanisms had a more preponderant role on ENR removal than on the removal of CEF, biodegradation is expected to have an important role in the environmental removal of both these antibiotics, even when present in a mixture. It was also possible to attest that environmental microbial communities have the capacity to adapt and respond to the presence of this type of contaminants, even in higher concentrations than those usually reported for antibiotics.

An important aspect of the work developed in this thesis was the investigation of the biodegradation of the target compounds when supplemented as mixtures of xenobiotics. In natural environments, contaminants are usually present in complex mixtures with other compounds, especially if they have similar sources of input into the environment. Mixtures of xenobiotics are relevant, as they can have increased deleterious effects in the environment due to synergetic interactions between them. Also, from a microbial point of view, the presence of the target compound in a complex mixture may alter metabolic dynamics, eventually

affecting overall biodegradation potential either due to metabolic inhibition or to toxic effects induced in the degrading microorganisms. This was observed in the study on the biodegradation of FAs, when MFA was fed in mixture with DFA, with microbial defluorination of the first compound decreasing markedly in the presence of the second, likely due to competitive substrate inhibition.

Acetate was used as a growth supporting substrate in the experimental work conducted in this thesis, for two main reasons: (i) to investigate the biodegradation of the target compounds in the presence of an easily accessible carbon and energy source that could serve as a cometabolite and (ii) to mimic the organic carbon loads usually present in some natural environments or in WWTPs. Cometabolism constitutes an important mechanism in the biodegradation of recalcitrant compounds, since the metabolic conversion of many of these compounds occurs through fortuitous reactions promoted by the presence of highly energetic substrates (Criddle, 1993). Also, in a real environmental scenario there is always organic matter available for microbial consumption.

2. Conclusion

The experiments conducted under the scope of this thesis showed that, although having increased resistance to biodegradation mechanisms when compared with other xenobiotics, fluoroorganic compounds can be metabolized by environmental microorganisms.

Several bacterial strains from distinct environmental sources were able to utilize MFA as a sole carbon source, though DFA and TFA were shown to be recalcitrant under different experimental conditions, indicating that the metabolic mechanisms involved in the biodegradation of MFA are not able to act in the degradation of these two compounds. The majority of these MFA-degrading bacterial strains have never been linked before to the biodegradation of this compound, and so this work shows for the first time the capacity of these microbial species to degrade this fluoroaliphatic. It was also found in this work that DFA negatively affects MFA microbial metabolism, which may be a limiting factor when considering the biological recovery of environmental matrices contaminated with mixtures of these compounds.

An enriched microbial consortium was capable of removing and defluorinating ENR supplied in a range of concentrations between 1 and 3 mg L^{-1}, though at different extents. Biodegradation of this compound markedly decreased with the increase in its concentration and was not affected by the concomitant presence of CEF. On the other hand, CEF biodegradation was not affected by the different concentrations tested. This study also revealed that the microbial communities used as inocula were capable of adapting and responding to the presence of these antibiotics. The results obtained indicate that bioremediation of environmental sites contaminated with mixtures of ENR and CEF may be possible, especially when assuming that antibiotic concentrations lower than those tested in this study are typically present.

In overall, these studies emphasized the potential of environmental-occurring microorganisms to biodegrade organofluorinated contaminants. Two main factors were identified as crucial in the biodegradability of the tested fluorinated compounds: the degree of fluorination and compound concentration. Microbial cultures used in the two conducted studies have potential to be used in bioremediation strategies of fluoroorganic compounds.

CHAPTER 5

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